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(54) Title: XYLOSYLTRANSFERASE AND ISOFORMS THEREOF

(57) Abstract: The invention relates to the isolation, purification and characterization of the enzyme xylosyltransferase (defined as "XT"). The invention describes for the first time that XT occurs in at least two isoforms ("XT-I", "XT-II"). The invention relates furthermore to the recombinant cloning and expression of human and rat XT-I and XT-II and discloses their DNA and protein sequences. The enzymes according to the invention can be used as therapeutic agents and as diagnostic markers, e.g. for the determination of enhanced proteoglycan biosynthesis, and as biochemical markers for determination of several pathological processes such as systemic sclerosis.

Xylosyltransferase and Isoforms thereof

The invention relates to the isolation, purification and characterization of the enzyme xylosyltransferase (defined as "XT"). The invention describes for the first time that XT occurs in at least two isoforms ("XT-I", "XT-II"). The invention relates furthermore to the recombinant cloning and expression of human and rat XT-I and XT-II and discloses their DNA and protein sequences. The enzymes according to the invention can be used as therapeutic agents and as diagnostic markers, e.g. for the determination of enhanced proteoglycyn biosynthesis, and as biochemical markers for determination of several pathological processes such as systemic sclerosis.

Background of the Invention

Proteoglycans are polyanionic molecules widely expressed in animal cells and virtually every tissue. These abundant molecules are present in the extracellular matrix and on the cell surface and serve a wide range of functions. They are increasingly implicated as important regulators in many biological processes, such as extracellular matrix deposition, cell membrane signal transfer, morphogenesis, cell migration, normal and tumor cell growth and viral infection (Ruoslahti, 1989, *J. Biol. Chem.* **264**, 13369-1372; Herold *et al.*, 1994, *J. Gen. Virol.* **75**, 1211-1222). Proteoglycans mediate diverse cellular processes through interaction with a variety of protein ligands. In most of these bindings electrostatic interactions with the glycosaminoglycan chains attached to the core protein are involved (Kjellen & Lindahl, 1991, *Annu. Rev. Biochem.* **60**, 443-475). Thus, the biological activity of proteoglycans is intimately related to the glycosaminoglycan biosynthesis.

The sulfated glycosaminoglycans chondroitin sulfate, heparan sulfate, heparin and dermatan sulfate are bound to the proteoglycan core protein by a xylose-galactose-galactose binding region (Kjellen & Lindahl, 1991, *l.c.*). UDP-D-xylose:proteoglycan core protein β -D-

various species (*Hoffmann et al.*, 1984, *Connect. Tissue. Res.* 12, 151-164), and it was shown that the enzyme is secreted from the endoplasmatic reticulum into the extracellular space together with chondroitin sulfate proteoglycans (*Kähnert et al.*, 1991, *Eur. J. Clin. Chem. Clin. Biochem.* 29, 624-625; *Götting et al.*, 1999, *J. Invest. Dermatol.* 112, 919-924). However, the
5 processes resulting in the release of XT from the endoplasmatic reticulum or the Golgi compartments and the role of XT in the extracellular matrix are not yet known .

As XT is the initial step enzyme in the biosynthesis of the glycosaminoglycan linkage region and as it is secreted into the extracellular matrix to a great extent, XT activity was proposed to
10 be a diagnostic marker for the determination of an enhanced proteoglycan biosynthesis and of tissue destruction (*Weilke et al.*, 1997, *Clin. Chem.* 43, 45-51). XT activities in the synovial fluid were found to be significantly increased in chronic inflammatory joint diseases (*Kleesiek et al.*, 1987, *J. Clin. Chem. Clin. Biochem.* 25, 473-481). Recent studies have shown that serum XT activity is a biochemical marker for the determination of fibrotic activity in systemic
15 sclerosis (*Götting et al.*, 1999, *l.c.*; *Götting et al.*, 2000, *Acta Derm. Venereol.* 80, 60-61.).

Up to now there was no success to isolate or characterize xylosyltransferase (XT), however some methods were described to measure and determine the activity of said enzyme from blood or body fluid samples of patients showing pathological effects such as scleroderma or chronic
20 joint diseases (*Stoolmiller*, 1972; *J. Biol. Chem.* 247, 3525-3532). The samples were incubated with UDP-[¹⁴C]xylose and an appropriate acceptor. The incorporated radioactivity indicated the amount of XT activity. Acceptors used so far are proteoglycans, silk fibroin (*Campbell et al.*, 1984, *Anal. Biochem.* 137, 505-516) and several peptides (*Bourdon et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84, 3194-3198). However, the all activity tests used herein did not allow precise
25 determination of the lower XT activity in serum. A more specific acceptor protein is recombinant bikunin, the inhibitory component of human inter- α -trypsin inhibitor. Bikunin carries a single chondroitin, which is essential for the structure of the inhibitor. The chondroitin sulfate attachment site in the N-terminal region contains all elements responsible for recognition by XT. The complete recognition sequence is composed of the amino acids a-a-a-a-
30 G-S-G-a-b-a, with a = E or D and b = G, E or D. This sequence was confirmed by determination of the Michaelis-Menten (K_m) constants for *in vitro* xylosylation of different synthetic proteins and peptides using an enriched XT preparation from conditioned cell culture

supernatant of human chondrocytes. The constant was determined to be 22 μ M, which was decreased 9-fold in comparison to deglycosylated core protein from bovine cartilage (188 μ M) (Brinkmann et al., 1997, *J. Biol. Chem.* 272, 11171-11175). With recombinant bikunin as acceptor, a sensitive assay was developed that allows precise determination of XT activity in human serum and other body fluids (*see*: Weilke et al., 1997, *l.c.*). Using this assay an increased xylosyltransferase activity was determined in blood of patients with sclerotic processes of scleroderma, closely related to an elevated proteoglycan biosynthesis (Götting et al., 1999, *l.c.*).

The biosynthesis of glycosaminoglycans requires the coordinated action of a large number of glycosyltransferases. Isolation and cloning of these glycosyltransferases has been targeted for a long time, since the majority of these enzymes are only present in minute amounts. The structure and sequence of the glycosyltransferases involved in biosynthesis of the common glycosaminoglycan-protein linkage region has long remained unknown. Recent cDNA cloning of galactosyltransferase I (Okajima et al., 1999, *J. Biol. Chem.* 274, 22915-22918) and glucuronyltransferase I (Kitagawa et al., 1998, *J. Biol. Chem.* 273, 6615-6618) identified 2 of the at least 4 enzymes involved in synthesis of the GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-O-Ser structure.

Isolation, purification and characterization of XT involved in biosynthesis of the common carbohydrate-protein linkage structure has been hampered by difficulties in obtaining a sufficient amount of the source material. Since, as above mentioned, XT can be used as an additional biochemical marker for the determination of sclerotic activity in systemic sclerosis and some inflammatory disorders, it is a real need for an isolated, highly purified XT which can be produced by recombinant methods, which is necessary for diagnostic and therapeutic purposes. Moreover, the knowledge of the cDNA sequence of XT allows to use it on gene level such as in gene diagnostic or gene therapy.

Definitions

- Above and below the term "XT" means xylosyltransferase (UDP-D-xylose:proteoglycan core protein β -D-xylosyltransferase (EC 2.4.2.26) deriving from any origin and includes all possible isoforms, if not otherwise pointed out. The term "hXT" has the meaning of human XT; the term "rXT" of rat XT. The terms "XT-I", "XT-II", "hXT-I", "hXT-II", "rXT-I2,

"rXT-II" mean the specific isoforms 1 and 2 of XT according to the invention, wherein h and r have the indicated meanings.

- Above and below the term "protein" means a protein, a protein fragment or a peptide, if not otherwise explained.
- 5 • Above and below the term "recombinant protein" is defined as a protein which was produced by recombinant and biotechnological methods.
- Above and below the term "XT" or "XT protein(s)" has the meaning of (a) protein(s) deriving from any source, if not otherwise stated out, having the biological activity and / or function of *UDP-D-xylose:proteoglycan core protein β -D-xylosyltransferase*.
- 10 • Above and below the term "a protein having the biological activity of UDP-D-xylose:proteoglycan core protein β -D-xylosyltransferase" is defined as a protein which has XT – identical or XT – like functions and activities and comprises XT itself and possible mutations, variants, isoforms thereof, including insertions, deletions and substitutions of one or more amino acids. The term includes also fragments or longer forms of XT as well as
- 15 dimeric or multimeric forms thereof showing XT functions.
- Above and below the term "isoform" means a second naturally occurring enzyme having the same biological activity as a first naturally occurring enzyme, however differing by another amino acid sequence.

20 Summary of the Invention

It was found that XT can be purified 4,700-fold with 1% yield from serum-free JAR choriocarcinoma cell culture supernatant. The isolation procedure includes a combination of ammonium sulfate precipitation, heparin affinity chromatography, ion exchange chromatography and protamine affinity chromatography. Amongst other proteins an unknown

25 protein was detected by matrix-assisted laser desorption ionization mass spectrometry-time of flight analysis (MALDI-TOF) in the purified sample. The molecular weight of this isolated and purified XT protein was determined as 120.000 by SDS-polyacrylamide gel electrophoresis. The isolated protein was enzymatically cleaved by trypsin and endoproteinase Lys-C. Peptide fragments were sequenced by Edman degradation. Searches with the amino acid sequences in

30 protein and EST databases showed no homology to known sequences. XT was enriched by immunoaffinity chromatography with an immobilized antibody against a synthetic peptide deduced from the sequenced peptide fragments and was specifically eluted with the antigen. In

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addition, XT was purified alternatively with an aprotinin affinity chromatography and was detected by western blot analysis in the enzyme-containing fraction.

Based on the partial amino acid sequence of their isolated and purified new enzyme (XT) derived from human JAR choriocarcinoma cell culture supernatant a novel cDNA was isolated according to the invention, encoding human XT-I enzyme using the degenerate reverse transcriptase-polymerase chain reaction method. The enzyme belongs to a novel family of glycosyltransferases having no homology to proteins of prior art. 5'- and 3'-RACE were performed to isolate a novel cDNA fragment of 3726 bp with a single open reading frame encoding at least 827 amino acids with a molecular mass of 91.000. The human XT-I gene was located on chromosome 16p13.1 using radiation hybrid mapping, and extracts from CHO-K1 cells transfected with the XT-I cDNA in an expression vector exhibited marked XT activity. Furthermore, a new 3608-bp cDNA fragment encoding a novel protein of 865 amino acids was also isolated by PCR using degenerate primers based on the amino acid sequence of human XT-I. The amino acid sequence of this XT-II isoform displays 55% identity to the human XT-I. The XT-II gene is located on chromosome 17q21.3-17q22, and the exon/intron structure of the 15 kb gene was determined. RT-PCR analyses of XT-I and XT-II mRNA from various tissues confirmed that both XT-I and XT-II transcripts are ubiquitously expressed in the human tissues, although with different levels of transcription. Furthermore, the cDNAs encoding XT-I and XT-II from rat were cloned. The deduced amino acid sequences of rat xylosyltransferases displayed 94% identity to the corresponding human enzyme.

Therefore, it is an object of this invention to provide the following subject-matters:

- An isoform of *UDP-D-xylose:proteoglycan core protein β -D-xylosyltransferase*(XT);
- a protein comprising a sequence of said isoform or a fragment thereof, having the biological activity of XT;
- a corresponding protein deriving from human or rat sources (hXT, rXT);
- a corresponding protein isolated from specific human tissue, wherein said hXT has a molecular weight of 120.000 under SDS PAGE conditions;
- a corresponding recombinant protein, wherein said protein is hXT-I comprising at least 827 amino acids and having the amino acid sequence as depicted in Fig. 7B;

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- a corresponding isoform of hXT-I, termed as hXT-II, comprising 865 amino acids, exhibiting approximately 55% overall sequence identity to human hXT-I, and having, in more detail, the amino acid sequence as depicted in Fig. 8B.
- a process for isolating and purifying a protein having the biological activity of human UDP-
5 D-xylose:proteoglycan core protein β -D-xylosyltransferase (EC 2.4.2.26), said process is characterized by the following steps:
 - (i) culturing from human tissue showing an enhanced XT activity, preferably from JAR choriocarcinoma cells (ATCC HTB-144), and harvesting the cell culture supernatant,
 - 10 (ii) fractionated ammonium sulfate precipitation of the supernatant of step (i),
 - (iii) heparin affinity chromatography of the precipitate of step (ii),
 - (iv) ion exchange chromatography of the step (iii) product, and
 - (v) affinity chromatography of the step (iv) product, and optionally
 - (vi) a SDS-Polyacrylamide gel electrophoresis of step (v);
- 15 • recombinant forms of hXT and rXT, the corresponding DNA sequences (Fig. 7A, 8A, 9, 10) included as well as suitable expression vectors and expression host cell systems;
- antibodies directed against any of the above or below mentioned XT proteins and their uses in immunological assays and diagnostic tools for determining said XT proteins;
- pharmaceutical compositions comprising a XT protein as defined above and below,
20 optionally together with a suitable pharmacologically acceptable carrier, diluent or excipient;
- uses of said XT proteins for the manufacture of a medicament for the treatment of XT relevant diseases and disorders, wherein the xylosyltransferase enzymes according to the invention can either be used directly as therapeutic drug in pathological situations where a
25 deficiency of said enzyme and its isoforms can be detected;
- uses of said XT enzymes for the manufacture of a medicament for the treatment of diseases and disorders which are caused or accompanied by increased levels of said enzymes, e.g. in sclerotic diseases and chronic joint diseases, wherein said medicament is an inhibitor or antagonist of said XT proteins;
- 30 • uses of said XT proteins as diagnostic markers for the diagnosis of above- and below-mentioned diseases and pathological symptoms and uses of said DNA molecules as gene markers;

- methods of screening for compounds which are capable to inhibit the activity of said XT proteins according to the invention using known methods for determining the activity of XT.

5 Detailed Description:

General

UDP-D-xylose: proteoglycan core protein β -D-xylosyltransferase can be isolated and purified from JAR choriocarcinoma cell culture. The isolated protein according to the invention is a single stranded polypeptide with a molecular weight of 120.000. The protein was enzymatically
10 cleaved and eleven peptide fragments were sequenced by Edman degradation (see Examples). XT is present only in very small amounts in animal tissues but unlike other glycosyltransferases, more than 90% of XT is enriched in the medium of cultured cells. The highest secretion of XT activity was measured in JAR choriocarcinoma cell culture, in which sternal cartilage chondrocytes and 21 different human cell lines were examined. To produce a
15 highly enriched XT solution for the isolation of XT, JAR choriocarcinoma cells were adapted to hollow fiber culture conditions using a novel bioreactor (TECNOMOUSE) and Ultradoma-PF medium without serum addition as nutrient. For purification of XT a combination of classic separation methods and new affinity matrices was employed. Therefore, a heparin matrix was used as an affinity ligand for the XT. When applied to immobilized heparin, XT was
20 completely adsorbed at the matrix and the XT activity was eluted only with a high salt concentration after most contaminating proteins have been removed from the matrix. Protamine chloride is well-known as cationic activator for several sulfotransferases, so the effect of protamine chloride on the XT was investigated. An increased XT activity was measured when protamine chloride was added to the XT assay solution (see Examples), indicating an
25 interaction of these arginine rich proteins with XT. Therefore, a protamine chloride affinity matrix was synthesized using an aldehyde activated perfusion medium as support. The interaction of XT with this affinity matrix resulted in a 13.6 times enrichment of XT. The protamine chloride affinity chromatography was the most efficient purification step during the isolation of the XT. Immobilized aprotinin, a Kunitz-type proteinase inhibitor, was found to be
30 another appropriate affinity matrix for enrichment of XT, as it was able to adsorb XT quantitatively. Therefore, it was used for alternative purification of XT. Different lines of evidence showed that the isolated protein corresponds to the XT: (a) XT activity was enriched

using immobilized antibodies raised against a synthetic peptide deduced from the 120.000 protein, and the XT activity could be competitively eluted with this peptide. (b) Immunoblot analysis of aprotinin affinity purified XT corresponds with the 120.000 protein. However, non-reducing and non-denaturing gel filtration chromatography with heparin affinity-purified XT from JAR cell culture supernatant shows an additional peak of XT activity at a molecular weight of approximately 500.000. XT is associated with proteoglycans. Treatment of XT with N-glycosidase F resulted in a decrease of the molecular weight from 120.000 to 116.000, suggesting that the XT is a glycoprotein. A comparison of the molecular mass of XT with other glycosyltransferases involved in biosynthesis of proteoglycans shows that the XT is larger than the other enzymes. Another difference is that nearly all proteoglycan glycosyltransferases are tightly bound to the membrane of the endoplasmic reticulum, whereas XT is secreted into the extracellular space. XT according to the invention contains like many glycosyltransferases a DxD motif, suggesting that this motif is involved in binding the metal-ion cofactor and the donator substrate (Gastinel et al., 1999, *EMBO J.* 18, 3546-3557). A DxD sequence was also found in peptide 8 obtained from the enzymatically cleaved XT.

The invention discloses for the first time the molecular cloning and expression of human and rat XT. Surprisingly, XT is found in at least two isomeric forms, which are termed according to the invention as XT-I and XT-II. Based on the amino acid sequence a novel cDNA was cloned, which encodes a protein of at least 827 amino acids with a molecular mass of 91.000. A DxD motif was identified in the XT-I amino acid sequence using hydrophobic cluster analysis. This motif has been observed in many glycosyltransferases, and is involved in the coordination of a divalent cation in the binding of the nucleotide-sugar (Breton & Imberty, 1999, *Curr. Opin. Struct. Biol.* 9, 563-571.). The translation initiation codon of the XT-I cDNA could not be yet cloned probably due to strong secondary structures in the 5'-region of the XT-I mRNA. The XT-I protein isolated from human JAR choriocarcinoma cell culture supernatant migrated on SDS-PAGE with a molecular mass of 120.000 and the protein size could be further reduced after N-glycanase digestion. These findings indicate that the cloned cDNA represents at least 90% of the coding region of human XT-I.

Another cDNA was completely identified from human placenta RNA, which was similar but not identical to the hXT-I cDNA. The new cDNA encodes a protein of 865 amino acids with a molecular weight of 97.000. This novel protein termed XT-I has a proline-rich region located

near the amino-terminus and the type II transmembrane topology characteristic of many other glycosyltransferases cloned to date. The hXT-II protein was similar to the human XT-I with an overall sequence identity of 55%. The similarity of the predicted amino acid sequences was low (<10%) at the amino-terminal end. However, large stretches at the C-terminal region, where the catalytic domain was found to be located in glycosyltransferases, are very conserved with an identity of more than 80% in both proteins. These findings let conclude that the XT-II gene encodes another human xylosyltransferase, although the catalytic activity and the biological role of XT-II remain to be elucidated in detail.

Since alterations in XT activity have been reported to be associated with fibrotic and sclerotic alterations of connective tissue (Götting *et al.*, 1999; Götting *et al.*, 2000, l.c.), the present findings provide molecular tools to study the function and the regulated expression of human XT as well as the molecular mechanisms of these diseases.

Production of XT

JAR choriocarcinoma cells which had been adapted to growth in the serum-free Ultradoma-PF medium secreted XT activity into the cell culture supernatant. During the exponential growth XT activity and total protein concentration in the supernatant of a traditional cell culture system (T-flasks) were determined as 0.2 mU/l and 0.1 g/l, respectively. The cells were cultivated using three "Tecnomouse"® bioreactor systems. Each bioreactor contained five culture cassettes. About 10^7 cells per culture cassette were inoculated and medium probes of about 0.5 ml were taken every day to determine the viability of the cells and the glucose, and lactate concentration as well as the XT activity of the cell culture supernatant. The cells amounted in the probes varies from 10^5 to 10^7 cells per ml with viability between 40 and 70%. XT production increased within three weeks after cell inoculation, reaching a plateau of approximately 30 mU/l. After three months the culture cassette was removed. Harvesting was carried out every two days, collecting 10 ml cell culture supernatant per culture cassette. The cell-free supernatant was collected and stored at -75°C. Mean XT activity in the supernatant of the high density culture was determined as 29.0 mU/l, while the total protein concentration was estimated as 4.8 g/l. In total 18.5 l high density cell culture supernatant was collected and yielded 535.8 mU XT.

Isolation and purification of XT from cell culture supernatant

XT was purified from 18.5 l supernatant (equivalent to 2.000 l normal cell culture supernatant) of serum-free cultivated JAR choriocarcinoma cells to an apparent homogeneity of about 4700-fold purification. A summary of the purification steps for isolation of XT is shown in Table 1.

- 5 XT activity of the crude supernatant was approximately 0.006 mU/mg protein. The purification method according to the invention comprises four, preferably five different steps. It is possible, that one step can be achieved more than once if necessary.

Step 1: Fractionated ammonium sulfate precipitation XT of the ammonium sulfate precipitable fraction was dissolved in 0.46 l buffer A with solubilization of 79.5% of the original activity.

- 10 Step 2: Heparin affinity chromatography on POROS 20 HE 4 ml of XT-enriched solution from step 1 was loaded onto the POROS 20 HE column. XT activity was completely retained on the column. More than 70% of total protein passed through the column. Contaminating protein was eluted at a low NaCl concentration. 44% of the XT activity bound to the heparin matrix emerged at 0.5 M NaCl (Fig. 1A).

- 15 Step 3: Ion exchange chromatography on POROS 20 HQ 4 ml of the desalted XT-containing fraction from step 2 was loaded onto the POROS 20 HQ column equilibrated in buffer A. More than 98% of the XT activity bound to the resin. The column was then eluted stepwise with NaCl in buffer A (Fig. 1B). XT-containing fractions were collected.

- Step 4: Affinity chromatography on protamine chloride The product of step 3 was desalted and
20 concentrated using dia- and ultrafiltration. 100 µl of the protein solution was applied to the POROS protamine chloride column previously equilibrated with buffer A. Approximately 95% of the transferase activity bound to the column, whereas 75% of the contaminating protein did not. Additional proteins were eluted with buffer A containing low NaCl concentrations. Enzyme activity was eluted at approximately 0.15 M NaCl (Fig. 1C). The enzyme activity
25 was stable for at least 6 months at -75°C.

- Step 5: SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) XT-containing fractions from step 1-4 were subjected to SDS-PAGE on a 4-12% gradient polyacrylamide gel (Fig. 2, panel A). Coomassie-stained protein bands were excised and characterized by MALDI-TOF mass spectrometry after tryptic digestion. The molecular weight of an unknown protein was
30 determined as 120.000 (Fig. 2, panel B).

Step 5 is an optionally step.

Table 1:

Summary of single purification steps employed for isolation of XT from 18.5 l of high density JAR cell culture supernatant.

Step	Volume ml	Total activity 10 ⁻³ x units	Total protein mg	Spec. activity 10 ⁻³ x units/mg	Purification -fold	Recovery %
JAR high density cell culture						
Supernatant	18,500	535.8	89,355.0	0.006	1	100
Ammonium sulfate precipitation	460	426.0	8,937.8	0.048	8	79
Heparin affinity chromatography	50	108.3	473.0	0.229	40	35
Ion exchange Chromatography	5	91.0	43.1	2.090	348	17
Protamine affinity chromatography	1	6.83	0.24	28.458	4,743	1

- 5 *Amino acid sequence analysis of XT.* The MW 120.000 protein from the excised band was digested with trypsin and endoproteinase Lys-C. The proteolytic fragments were separated by reversed-phase HPLC, and selected peptides were subjected to automatic amino acid sequence analysis. Table II shows the obtained 11 amino acid sequences determined by Edman degradation and mass spectrometry.
- 10 Table II. X represent a not identified residue. The masses of three peptides were observed in the MALDI mass spectrum of the enzymatically digested MW 120.000 protein. Calculated mass values were obtained from the sequence obtained.

Peptide	observed mass (M + H ⁺)	calculated mass (M + H ⁺)
(1) ELGAK		
(2) ELLK		
(3) DMNFLK		
(4) IASPPSDFGR	1045.5	1045.5
(5) LLLD		
(6) DFENVDNSNFAPR	1524.7	1525.7
(7) PTFFAR		
(8) LQFSEVGTDXDA		
(9) ELGAVKPDGRL	1152.6	1153.7
(10) ELLKRKLEQQEK		
(11) LGLLMPEK		

Immunochemical detection of XT

Polyclonal antibodies against the synthetic peptide CSRQKELLKRKLEQQEK deduced from the peptides 2 and 10 of the enzymatically cleaved MW 120.000 protein were covalently bound on POROS 20 PA. About 50% of the XT activity of an applied sample was bound (Fig. 3, panel A) when a partially purified XT sample obtained by heparin affinity chromatography (purification step 2) was loaded onto the column. 58% of the adsorbed XT activity was eluted with 150 mM NaCl, and the rest was eluted with 12 mM HCl. Furthermore, the adsorbed XT activity was also eluted from the solid phase when 100 µl (1 mg/ml) of the synthetic peptide was added to the mobile phase (Fig. 3, panel C). When immobilized preimmune serum was used as affinity matrix (negative control) no XT activity was adsorbed to or eluted from the matrix (Fig. 3, panel B). The desalted XT fraction after heparin affinity chromatography (purification step 2) was loaded on an aprotinin affinity column. The elution profile shows four major protein peaks (Fig. 4, panel I). 61% of the XT activity adsorbed to the aprotinin matrix emerged at 0.30 M NaCl and another 21% at 0.54 M NaCl. A single MW 120.000 band of the XT-containing fractions was detected by western blot analysis with the polyclonal antibodies (Fig. 4, panel B).

Determination of the molecular weight of XT

100 µl of heparin affinity purified XT was separated under non-reducing and non-denaturing conditions using a TSK G3000 SW column. Two XT activity maxima were detected at MW 500.000 and 120.000 (Fig. 5, panel A). The molecular mass of the MW 120.000 protein was reduced about 3 % after N-glycosidase F digestion as shown by SDS-PAGE (Fig. 5, panel B).

PCR-based cloning of human XT-I cDNA

Based upon the amino acid sequence of 4 peptides degenerate primers were designed for cloning the XT-I cDNA (Figure 6). When primers SPPS1 and Lysc-inv were used in a PCR with the first strand cDNA of SW1353 chondrosarcoma poly(A)⁺ mRNA as template, a major band of approximately 690 bp was observed. After subcloning and sequencing a previously unknown DNA sequence was obtained from clone pCG114-29. PCR amplification with the primers DF1 and a mixture of Inv2b and Inv2c resulted in a 1724 bp fragment, which was subcloned and sequenced. The deduced amino acid sequence of the clone pCG111-4 was identical to 6 of the sequenced peptides from human XT-I. The cloning strategy of rapid

amplification of cDNA ends (RACE) (*Chenchik et al., 1996, BioTechniques 21, 526-534*) was employed to clone the complete coding sequence of the XT-I cDNA. The largest DNA fragment obtained from the 3'-RACE reaction was 1.6 kb and consisted of a 3'-untranslated region of 1240 bp. The 5' RACE reaction was performed with different primers derived from the nucleotide sequence of XT-I cDNA, but the 5'-untranslated region of human XT-I cDNA could not be cloned using this method. All DNA fragments obtained contained just the known cDNA sequence and an additional 80 bp of coding sequence. Thus, a PCR-based screening approach using cDNA libraries as template was employed for cloning of the 5'-untranslated region. All DNA fragments obtained from the screening of 3 different human cDNA libraries stopped at the same nucleotide, indicating that stable secondary structures of the XT-I mRNA prevent the synthesis of cDNA of the 5' untranslated region during the reverse transcription reaction. However, the translation initiation codon of human XT-I could not be yet cloned. The combined cDNA of human XT-I contained 3726 bp (Fig. 7A) with a single open reading frame encoding at least 827 amino acids with a molecular mass of a least 91.000. The deduced amino acid sequence contained 3 potential N-glycosylation sites (Figure 7B). Analysis of the amino acid sequence using the hydrophobic cluster analysis (*Gaboriaud et al., 1987,*) revealed the presence of a common DxD motif at position 182, which has been shown to be essential for binding nucleotide-sugars in glycosyltransferases .

20 *PCR-based cloning of human XT-II isoform cDNA*

The degenerate primers PFF-sense and Lysc-inv1 which were designed upon the amino acid sequence of proteolytic cleaved peptides of human XT-I were used in a PCR reaction with first strand cDNA of placenta poly(A)⁺ mRNA as template. The PCR amplification resulted in a minor band of 1.1 kb, which was cloned and sequenced. The determined nucleotide sequence was similar but not identical to the XT-I cDNA sequence, indicating that the fragment encodes a XT-II isoform. To clone the complete coding sequence of the novel cDNA, the RACE strategy was employed. The cDNA finally obtained was 3608 bp (Figure 8A) and contained a single open reading frame encoding a protein of 865 amino acids with a molecular mass of 97.000. The 3'-untranslated region is 850 bp and a 5'-untranslated region of 149 bp was identified with an in-frame stop codon upstream of the ATG codon. A Kyte-Doolittle hydrophathy analysis (*Kyte & Doolittle, 1982, J. Mol. Biol. 157, 105-132*) of the deduced amino acid sequence revealed one potential membrane-spanning region consisting of 16 hydrophobic amino acid residues at position 16 to 32, which appears to result in a type II transmembrane

orientation characteristic of many of the glycosyltransferases. The predicted amino acid sequence contains a proline-rich profile pattern from position 110 to 118 and 3 potential N-glycosylation sites (Figure 8B). The human XT-II isoform protein exhibits 55% overall sequence identity to the human XT-I including stretches in the XT-II protein with more than
5 80% homology to the human XT-I.

Cloning of rat XT-I and XT-II cDNA

For amplification of the XT-I and XT-II cDNA from rat tissue gene-specific primers were
10 designed based upon the nucleotide sequence of human XT-I and XT-II cDNA. These oligonucleotides were employed in PCR, 5' RACE and 3' RACE reactions with rat liver cDNA as template and a 2593 bp cDNA fragment (Fig. 9) coding for rat XT-I was obtained. 5' RACE reactions using a diversity of gene-specific primers were performed for identification of the 5' end of the XT-I cDNA. All cDNA fragments obtained stopped at exactly the same nucleotide
15 position as observed in the human XT-I cDNA, indicating that stable secondary structures of the XT-I mRNA inhibit cDNA synthesis of the 5' region. The XT-II cDNA fragment finally obtained after PCR amplification with rat liver cDNA was 2782 bp (Fig. 10) and included the entire coding region of rat XT-II. The deduced amino acid sequences of XT-I and XT-II (SEQ IDs 6, 8) from rat each displayed 94% identity to the corresponding human xylosyltransferase.
20 Alignment of all amino acid sequences revealed the presence of highly conserved amino acid clusters in the central and carboxyterminal regions of the proteins.

Identification of the chromosomal localization of the human XT-I and XT-II genes

The gene coding for human XT-I could be assigned to the short arm of chromosome 16 at the
25 region 16p13.1. The radiation hybrid mapping using the Genebridge 4 radiation hybrid screening panel located the position of the PCR fragment 5.98 cR distal to the STS marker CHLC.GATA42E11 and 4.40 cR proximal to marker D16S499. The XT-II gene was identified on chromosome 17 at the position 17q21.3-17q22 and was found to be located distal to the STS marker WI-11424 and proximal to the marker WI-14315.

30

Expression of recombinant XT

To prove the function of the cDNA product, a recombinant form of XT-I was generated by fusing the cloned XT-I to an aminoterminal peptide tag. The fused protein was expressed in

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CHO-K1 cells and absorbed from the medium by immunoprecipitation with anti-*Xpress*-antibodies and protein G agarose beads to eliminate endogenous XT activity. The enzyme-bound beads were used as an enzyme source and assayed for XT activity as shown in Table 3 (see below). No detectable XT activity was recovered by the affinity purification from a control
5 transfection sample. The substrate specificity of the recombinant XT-I was similar to that of the XT-I isolated from human body fluids and cell culture supernatants. The recovered enzyme activity of the recombinantly expressed XT-I could be completely inhibited by addition of 250 U of heparin. As specific inhibition of human XT activity by heparin has been demonstrated previously (Kleesiek *et al.*, 1987, l.c.), these results clearly indicate that the expressed protein is
10 the human XT. However, no enzymatic transfer of xylose to the acceptor peptides used in this study was observed when expressing XT-II fused to the aminoterminal peptide tag in CHO-K1 cells.

To identify the XT-I reaction products, the bikunin peptide was labeled with [¹⁴C]-D-xylose using the XT-I-bound beads as an enzyme source. The products were subsequently subjected to the linkage-specific digestion of the bound [¹⁴C]-D-xylose with α - and β -xylosidase and alkaline β -elimination. Incubation of the reaction products with β -xylosidase resulted in the release of 74% of the incorporated [¹⁴C]xylose, whereas only less than 4% of the peptide-bound xylose was digested after treatment with α -xylosidase. The alkaline cleavage of the O-
20 glycosidic linkage between the xylose and the β -hydroxyamino acid serine in the presence of borohydride lead to the liberation of more than 97% of the enzymatically transferred [¹⁴C]xylose. In all the experiments performed no significant amount of [¹⁴C]-D-xylose was incorporated without the addition of the bikunin peptide as acceptor. These results clearly indicate that a xylose residue was transferred to the hydroxyamino acid serine of the bikunin
25 peptide through a β -linkage. In conclusion the expressed protein was identified as UDP-D-xylose:proteoglycan core protein β -D-xylosyltransferase (EC 2.4.2.26).

Table 3:

Xylosyltransferase activity of recombinant XT-I expressed in CHO-K1 cells. XT activity of the enzyme fractions using different acceptors and the inhibition of enzyme activity by addition of heparin is shown. The synthetic bikunin, L-APP and L-APLP2 homologous peptides have
30 been previously proved to be good acceptors for XT mediated xylosylation (Brinkmann *et al.*, 1997; Götting *et al.*, 1998). No XT activity was detected in samples from mock-transfected cells after affinity purification. n.d., not detected (detection limit, 20 μ U/l).

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Acceptor	XT Activity	
	pCG227-XT	+ heparin
Recombinant bikunin	2854	n.d.
Bikunin peptide QEEGSGGGGQK	463	n.d.
L-APLP2 peptide SENEGSGMAEQK	515	n.d.
L-APP peptide TENEGSGLTNIK	492	n.d.
Peptide SGG	n.d.	n.d.
Chondroitin sulfate A	n.d.	n.d.
Chondroitin sulfate C	n.d.	n.d.

Tissue-specific expression of XT-I and the XT-II isoform

The expression of XT-I and XT-II isoform gene was examined in various human tissues using a RT-PCR based method with normalized first-strand cDNA. Each PCR yielded a single product
 5 with predicted nucleotide lengths of 490 bp for XT-I and 717 bp for XT-II, although the amount of the amplified product varied (Figure 11). Amplification of XT-I and XT-II fragments resulted in a product visible by ethidium bromide staining after 36 cycles, whereas the DNA fragment corresponding to the abundant glyceraldehyde-3-phosphate dehydrogenase transcript was visible after 20 cycles. The greatest abundance of XT-I expression was detected in the
 10 placenta, kidney and pancreas and only a very weak expression was detected in skeletal muscle. The greatest abundance of XT-II isoform is expressed in the kidney and pancreas.

Cellular distribution of XT activity in cultured CHO-K1 cells

After an incubation period of 3 days cultured CHO-K1 cells were harvested and the XT activity
 15 was determined in the spent culture supernatant and the cell lysates. 92% of the total XT activity was found to be located in the cell culture medium (93.1 mU/10⁶ cells, SD 9.58), whereas only 2% was detected in the cell lysates (2.03 mU/10⁶ cells, SD 0.44). 6% of the total XT activity (5.82 mU/10⁶ cells, SD 2.18) was released from the membrane-containing fractions after addition of the detergent Triton X-100 indicating that less than 1/10th of XT is bound to
 20 cellular membranes.

Pharmaceutical and diagnostic use

The xylosyltransferase enzymes according to the invention can be used directly as therapeutic drug in pathological situations where a deficiency of said enzyme and its isoforms can be
 25 detected. As pointed out above XT enzyme may be overexpressed in some diseases and

disorders. In these cases inhibitors and / or antagonists of XT-I and or XT-II can be used. Thus, the invention relates also to the use for the manufacture of a medicament for the treatment of diseases which are caused by increased levels of said enzymes, wherein said medicament is an inhibitor or antagonist of xylosyltransferase.

- 5 As mentioned above the protein according to this invention can be used as diagnostic means to evaluate pathological conditions. As used herein, the term "pharmaceutically acceptable carrier" means an inert, non toxic solid or liquid filler, diluent or encapsulating material, not reacting adversely with the active compound or with the patient. Suitable, preferably liquid carriers, are well known in the art. The formulations according to the invention may be administered as unit
10 doses containing conventional non-toxic pharmaceutically acceptable carriers, diluents, adjuvants and vehicles which are typical for parenteral administration.

The term "parenteral" includes herein subcutaneous, intravenous, intra-articular and intratracheal injection and infusion techniques. Also other administrations such as oral administration and topical application are suitable. Parenteral compositions and combinations
15 are most preferably administered intravenously either in a bolus form or as a constant fusion according to known procedures. Tablets and capsules for oral administration contain conventional excipients such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, and wetting agents. The tablets may be coated according to methods well known in the art.

- 20 Unit doses according to the invention may contain daily required amounts of the protein according to the invention, or sub-multiples thereof to make up the desired dose. The optimum therapeutically acceptable dosage and dose rate for a given patient (mammals, including humans) depends on a variety of factors, such as the activity of the specific active material employed, the age, body weight, general health, sex, diet, time and route of administration, rate
25 of clearance, enzyme activity (units/mg protein), the object of the treatment, i. e., therapy or prophylaxis and the nature of the disease to be treated.

Therefore, in compositions and combinations in a treated patient (in vivo) a pharmaceutical effective daily dose of the protein of this invention (hXT, hXT-I, hXT-II) is between about 0.01 and 100 mg/kg body weight (based on a specific activity of 100 kU/mg), preferably between
30 0.1 and 10 mg/kg body weight. According to the application form one single dose may contain between 0.5 and 10 mg of hXT.

Short Description of the Figures

Figure 1: *Purification of XT*

Panel A, Heparin affinity chromatography on POROS 20 HE: The dissolved ammonium sulfate precipitate from JAR choriocarcinoma supernatant was applied to a POROS 20 HE column. After equilibration with buffer A, the column was eluted stepwise with NaCl (----). Protein elution was monitored at wavelength A_{280} (—), and fractions of 38 ml were assayed for XT activity (■). The horizontal bracket indicates fractions collected for further purification.

Panel B, Ion exchange chromatography on POROS 20 HQ: The desalted and concentrated product from heparin affinity chromatography was loaded onto a POROS 20 HQ column. The column was washed with buffer A. Adsorbed proteins were eluted stepwise with NaCl (----). Protein elution was monitored at A_{280} (—), and fractions of 50 ml were assayed for XT activity (■). The horizontal brackets indicate the fractions collected for further purification.

Panel C, Protamine chloride affinity chromatography: The desalted and concentrated product from ion exchange chromatography was applied to a protamine chloride POROS column. After a washing step with buffer A, the column was eluted stepwise with NaCl (----). Elution was monitored at A_{280} (—), and fractions of 6 ml were assayed for XT activity (■). The horizontal bracket indicates the fractions collected.

Figure 2 *SDS-PAGE of XT fractions at various purification steps*

Panel A: XT fractions at various purification steps were subjected to 4-12% gradient polyacrylamide gel for SDS-PAGE. *Lane I*, JAR cell culture supernatant (crude material); *lane II*, ultrafiltration retentate; *lane III*, dissolved ammonium sulfate precipitate; *lane IV*, protein eluted with NaCl from the POROS 20 HE column; *lane V*, protein eluted with NaCl from the POROS 20 HQ column; *lane VI*, protein eluted with NaCl from the protamine chloride POROS column. *Lane M*, molecular size standard: myosin (200.000), phosphorylase b (97.000), bovine serum albumin (66.000), glutamic dehydrogenase (55.000), carbonic anhydrase (31.000), trypsin inhibitor (22.000). The gel was silver-stained.

Panel B: Collected XT fractions from protamine affinity chromatography were subjected to SDS-PAGE on a 4-12% gradient polyacrylamide gel. The arrows indicate the stained bands corresponding to the unknown protein, which was shown to be XT (120.000) (1), hexose-6-phosphate dehydrogenase (89.000) (2), ezrin (68.600) (3), quiescin Q6 (64.000) (4), plasminogen activator (47.000) (5), aldolase A (39.000) (6) and low molecular artefacts (7). All

protein bands were excised, enzymatically digested, and the peptide mixture was characterized by MALDI-TOF mass spectrometry. The gel was stained with Coomassie Brilliant Blue.

Figure 3: *Immunoaffinity chromatography of xylosyltransferase*

Panel A, 100 µl of the desalted XT-containing fractions eluted from heparin affinity matrix was applied to a column of immobilized polyclonal antibodies. After washing with buffer D, the column was eluted as indicated by the arrow with buffer D / 0.15 M NaCl (1) followed by 12 mM HCl (2). Fractions of 1 ml were collected into tubes containing 1 ml of 0.1 M Tris/HCl, pH 8.0. Protein elution was monitored at 280 nm (—) and the XT activities (■) of each fraction were assayed.

Panel B, Negative control of the immunoaffinity chromatography with immobilized preimmune serum. For conditions see *Panel A*.

Panel C, Immunoaffinity chromatography with immobilized polyclonal antibodies. The column was eluted with buffer D containing 100 µl (1 mg/ml) of the peptide antigen indicated by the arrow. Fractions of 1 ml were collected into tubes containing 1 ml of 0.1 M Tris/HCl, pH 8.0. Protein elution was monitored at 280 nm (—) and the XT activities (■) of each fraction were assayed.

Figure 4: *Aprotinin affinity chromatography of partially purified XT and immunoblot analysis of the separated fractions*

Panel A, 200 µl desalted XT fraction from the heparin purification step was applied to an aprotinin column previously equilibrated with buffer A. After washing with buffer A, the adsorbed proteins were eluted stepwise with NaCl (---). Protein elution was monitored at A₂₈₀ (—) and fractions of 2 ml were assayed for XT activity (■).

Panel B, Aliquots of fraction 3, fraction 7, fraction 12 and fraction 16 were analyzed by western blot. The MW 120.000 protein was detected in the XT-containing fraction 7 and 12. Immunological detections were performed using a polyclonal rabbit antiserum raised against the synthetic peptide CSRQKELLKRKLEQQEK deduced from the peptides 2 and 10 of the enzymatically cleaved unknown protein. Prestained molecular size standard were myosin (190.000), BSA (64.000), glutamic dehydrogenase (51.000).

Figure 5: *Gel filtration chromatography and N-glycosidase F digestion*

Panel A, 100 µl of the XT containing fraction from heparin affinity HPLC was applied to a TSK G3000 SW column (30 cm X 7.5 mm, 10 µm particle size) previously equilibrated with buffer A / 0.15 M NaCl. Elution was performed with the same buffer. Fractions of 200 µl were collected and assayed for XT activity (■). Protein elution was monitored at A₂₈₀ (—). The

arrows indicate the elution positions of thyroglobulin (669.000) (1), ferritin (440.000) (2), aldolase (158.000) (3), albumin (67.000) (4), ovalbumin (43.000) (5), chymotrypsinogen A (25.000) (6), and ribonuclease A (13.700) (7).

Panel B. An aliquot of 1 µg of the MW 120.000 protein was analyzed by SDS-PAGE

- 5 (I). Aliquots (1 µg) of the MW 120.000 protein were digested with $3,1 \times 10^{-3}$ units of N-glycosidase F at 37°C for 1 h (II) and 12 h (III). The samples were then subjected to SDS-PAGE, and protein bands were detected by silver staining. The molecular size standard were myosin (200.000), b-galactosidase (116.000), phosphorylase b (97.000).

Figure 6: *Cloning strategy for human XT-I and XT-II isoform.*

- 10 (A) The peptides 4, 6, 7, and 9 are sequences that were most favorable for the design of degenerate PCR primers. The amino acid sequence of the peptides was obtained after proteolytic digestion of the purified human XT. The strategy for cloning of XT-I cDNA (B) and XT-II isoform (C) is illustrated. The open reading frame of XT-I and XT-II is shown as a filled box, and the location of the peptides 4, 6, 7 and 9 is illustrated by open boxes. The location and
15 orientation of the degenerate primers employed for cloning of XT-I and XT-II are marked by arrows. XT-I and XT-II cDNA inserts contained within the indicated plasmids were obtained using RT-PCR with degenerate primers (pCG111-4, pCG114-29', pCG110-7), 5' RACE (pCG185-21, pCG212-19, pCG319-23, 3' RACE (pCG204-38, pCG211-4) and RT-PCR with gene-specific primers (pCG176-1). H = A + C + T, Y = C + T, R = A + G, N = A + G + C + T, I
20 = deoxyinosine.

Figure 7A, B: *Nucleotide (A) and deduced amino acid (B) sequence of the hXT-I.*

The position of the peptide sequences obtained by digestion of the purified human XT are underlined (B). Potential N-glycosylation sites are double underlined. The DxX motif is intensified depicted. The figures are identical with SEQ ID 1 and 2.

- 25 **Figure 8A, B:** *Nucleotide (A) and amino acid (B) sequence of the hXT-II isoform.*

Potential N-glycosylation sites are double underlined (B). The putative transmembrane domain is underlined. The figures are identical with SEQ ID 3 and 4.

Figure 9: *Nucleotide sequence of rXT-I (SEQ ID 5).* The corresponding protein sequence is depicted in SEQ ID 6.

- 30 **Figure 10:** *Nucleotide sequence of rXT-II (SEQ ID 7).* The corresponding protein sequence is depicted in SEQ ID 8.

Figure 11: *Differential expression of the XT-I, XT-II gene in human tissues.* Semiquantitative RT-PCR with normalized first-strand cDNA was used to examine the abundance of XT-I and

XT-II transcripts. A 983 bp fragment of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified as control (A). The 490 bp XT-I cDNA fragment (B) and the 717 bp fragment amplified from XT-II isoform cDNA (C) were detected in each tissue indicating that both enzymes are ubiquitously expressed. The arrows indicate the expected position for each PCR product.

The following examples describe the invention in more detail without to limit scope of the technical teaching.

Example 1: *Materials for Isolation and Purifications.* Human JAR choriocarcinoma cells were purchased from ATCC (Rockville, MD). Dried UltraDOMA-PF medium was obtained from BioWhittaker (Vervier, Belgium) and *aqua ad injecta* from Braun (Melsungen, Germany). Heat-inactivated fetal calf serum, Dulbecco's phosphate-buffered saline, antibiotic/antimycotic solution, trypsin-EDTA solution, trypan blue, protamine chloride and the *Bicinchoninic Acid Protein Assay Kit* were purchased from Sigma (Deisenhofen, Germany). Cell culture flasks, serological pipettes, and sterile tubes were purchased from Becton Dickinson (Heidelberg, Germany). The hybrid hollow-fiber bioreactor TECNOMOUSE[®] was supplied by Integra Biosciences (Fernwald, Germany), the ACA analyzer by Dade Diagnostica (München, Germany) and the Super G analyzer by RLT (Möhnesee, Germany). UDP-[¹⁴C]xylose (9.88 kBq / nmol) came from DuPont (Bad Homburg, Germany), 25 mm diameter nitrocellulose discs from Sartorius (Göttingen, Germany), scintillation mixture and the liquid scintillation counter LS500TD was obtained from Beckman Coulter (Fullerton, CA). Ultrafiltration cells, YM1 membranes and PVDF membranes (Immobilon P) were purchased from Millipore (Eschborn, Germany). The chromatography media POROS 20 HQ, POROS 20 HE2, POROS 20 AL, POROS 20 EP and the HPLC workstation *Biocad Sprint* were supplied by Perseptive Biosystems (Framingham, MA). The gel filtration column TSK G3000 SW (30 cm x 7.5 mm, 10 µm particle size) was obtained from TosoHaas (Montgomeryville, PA). The MALDI mass spectrometer Reflex II was from Bruker Daltonik GmbH (Bremen, Germany) and protein sequencer Procise 494 cLC was purchased from PE Biosystems (Framingham, MA). Precast polyacrylamide gels, buffers, and NuPAGE electrophoresis system XCell II Mini-Cell and Blot Module were from Novex (San Diego, CA). The synthetic peptide CSRQKELLKRKLEQQEK and the rabbit antiserum were purchased from BioScience (Göttingen, Germany). Peroxidase-conjugated affinipure F(ab')₂ fragment goat anti-rabbit IgG (H+L) was purchased from

Dianova (Hamburg, Germany). N-glycosidase F was obtained from Roche (Mannheim, Germany).

Example 2: *Cell culture.* JAR choriocarcinoma cells releasing XT in the cell culture supernatant were cultured in Ultradoma-PF medium containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. After incubation for 24 h in the serum-containing medium, the cell cultivation was adapted to serum-free conditions as described previously (6). Scaling up of XT production was carried out in three hybrid hollow-fiber bioreactors TECNOMOUSE®. During the exponential growth the cells from three 175 cm² T-flasks (> 3 x 10⁷ cells) were detached with 0.5% trypsin and 0.2% EDTA in Dulbecco's phosphate-buffered saline by incubation at 37°C for 10 min. After centrifugation (5 min, 1000 x g) of the cell suspension the cell pellet was resuspended in 10 ml 37°C warm serum-free and protein-free Ultradoma-PF medium and washed three times with 20 ml of the same medium. The cell suspension was drawn into a 10 ml syringe and then inoculated into the extracapillary space (EC space) of the reactor. The bioreactor was connected with a 2 liter medium bottle and set to 150 ml/h in the recirculation mode, and the oxygenation pump was set as described in the operating manual. Five days after inoculation a 10 ml syringe was connected to the left hand EC port and 10 ml of cell culture supernatant was harvested from the EC space. The harvesting was continued every two days over a period of 3 months. Glucose and lactate concentration of the cell culture supernatant were controlled using the Super G analyzer and the ACA analyzer, respectively. The 2 liter medium bottle was replaced every three days. The viability of the cells was determined by trypan blue exclusion.

Example 3: *Synthesis of the protamine affinity matrix.* Protamine chloride was immobilized as ligand on POROS 20 AL. 30 mg ligand was dissolved in 10 ml of 10 mM phosphate / 0.15 M NaCl, pH 7.4. After the protein had been dissolved, 5 ml of 100 mM phosphate / 1.50 M NaCl, pH 7.4 was added. NaCNBH₃ was dissolved in the ligand/buffer solution to a final concentration of 5 mg/ml and 1.0 g POROS 20 AL was suspended in the same solution. The suspension was mixed gently on a shaker for 1 minute at RT. An additional 2 ml of 100 mM phosphate / 1.50 M NaCl, pH 7.4 was added to the suspension and the mixture was shaken continuously. This step was repeated every five minutes until the mixture volume was 25 ml. After additional shaking for 2 h, the medium was filtered on a sintered glass funnel. The matrix was suspended in 20 ml of 0.2 M Tris/HCl / 5 g/l NaCNBH₃, pH 7.2 and mixed gently on a shaker for 30 min at RT. After the media had been washed in a sintered glass funnel using 100

ml of 10 mM phosphate, pH 7.4, 100 ml of 1.0 M NaCl and another 100 ml of 10 mM phosphate, pH 7.4, the matrix was packed in a PEEK column (4.6 x 50 mm).

Example 4: *Synthesis of the aprotinin affinity matrix.* Aprotinin affinity matrix was synthesized according to the synthesis of the protamine affinity matrix, but using aprotinin as ligand. After
5 immobilization of the ligand the matrix was packed in a PEEK column (4.6 x 50 mm).

Example 5: *Purification of xylosyltransferase from JAR cell culture supernatant.*
Fractionated ammonium sulfate precipitation and chromatography steps were performed at RT, ultrafiltration and diafiltration were carried out at 4°C. 18.5 liters JAR cell culture supernatant collected from three hybrid hollow-fiber bioreactors, TECNOMOUSE®, each containing 5
10 culture cassettes, was concentrated to 800 ml with ultrafiltration cells using YM1 cellulose membranes. The retentate was centrifuged at 4,000 x g for 1 h. The supernatant was decanted, and the pellet was discarded.

Step 1: *Fractionated ammonium sulfate precipitation* - Solid ammonium sulfate was added to the supernatant to 28% saturation. After 1 h at RT the suspension was centrifuged at 4,000 x g
15 for 2 h, the supernatant was decanted, and the precipitate was removed. Additional ammonium sulfate was added to the solution to the point of 40% saturation, and the suspension was allowed to stand for 1 h. To recover the precipitate the supernatant was decanted after the suspension was centrifuged at 4,000 x g for 2 h. Before chromatography on immobilized heparin the precipitate was dissolved in 460 ml buffer A (20 mM sodium acetate, pH 6.0).

Step 2: *Heparin affinity chromatography on POROS 20 HE2* - The step 1 product was passed through a 0.2 µm filter. 4.0 ml of the filtrate was applied to a POROS 20 HE2 column (16 x 100 mm) equilibrated with buffer A at a flow rate of 40 ml/min. After washing the column with 100 ml of buffer A the XT activity was eluted with the same buffer containing NaCl. The NaCl concentration was increased stepwise: 20 ml buffer A / 0.09 M NaCl; 20 ml buffer A / 0.15 M
25 NaCl; 30 ml buffer A / 0.24 M NaCl; 24 ml buffer A / 0.30 M NaCl; 24 ml buffer A / 0.60 M NaCl; 24 ml buffer A / 1.00 M NaCl; and 24 ml buffer A / 1.89 M NaCl. Fractions of 38 ml each were collected and the XT activity was measured. The procedure was repeated 115 times by cyclic chromatography and the fractions containing XT activity (115 x 38 ml) were collected.

Step 3: *Ion exchange chromatography on POROS 20 HQ* - Collected fractions from step 2 were
30 desalted using diafiltration with YM1 cellulose membranes and ultrafiltration cells. After concentration of the desalted protein solution to 0.05 liter using analogous techniques the XT-enriched solution was subjected to ion exchange chromatography. 4.0 ml of the XT solution

was applied onto the POROS 20 HQ column (16 x 100 mm) previously equilibrated with buffer A at a flow rate of 40 ml/min. The column was washed with 80 ml buffer A, and the adsorbed protein was eluted stepwise using the same buffer containing 0.07 M NaCl (88 ml), 0.18 M NaCl (120 ml), and 0.36 M NaCl (120 ml) followed by a linear gradient of 0.36 – 1.00 M NaCl (200 ml) and another step of buffer A / 2.0 M NaCl (120 ml). 50 ml fractions were collected and assayed for activity and evaluated by SDS-PAGE. Chromatography was repeated 13 times, and the fractions exhibiting XT activity (13 x 50 ml) were collected for affinity chromatography.

Step 4: Affinity chromatography on protamine chloride - XT-containing solution from step 3 was desalted as described above and concentrated to 5 ml by ultrafiltration with YM1 cellulose membranes. The ultrafiltration product was passed through a 0.2 µm filter. 100 µl of the filtrate was loaded onto a protamine chloride - POROS column (4.6 x 50 mm) equilibrated with buffer A. The flow rate was 10 ml/min. The column was washed with 10.0 ml of buffer A, and the adsorbed fraction was eluted with the same buffer containing NaCl by a stepwise increase of the NaCl concentration: 6.6 ml buffer A / 0.04 M NaCl; 6.6 ml buffer A / 0.06 M NaCl; 6.6 ml buffer A / 0.23 M NaCl followed by a linear gradient of 0.23 – 1.20 M NaCl (4.2 ml) in buffer A. Fractions of 6.0 ml were collected, assayed for XT activity and evaluated by SDS-PAGE. Cyclic chromatography was repeated 50 times. The purified enzyme was collected, concentrated to 1.0 ml using ultrafiltration techniques and stored at – 75 °C.

Step 5: SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) - The protein composition of various fractions was estimated by SDS-PAGE. Briefly, 12.1 µl of sample was added to 4.7 µl of sample buffer (1.00 M Tris/HCl / 1.17 M sucrose, 0.28 M SDS, 2.08 mM EDTA, 0.88 mM Serva Blue G250, 0.70 mM phenol red, 0.10 M DTT, pH 8.5) and heated for 10 minutes at 99°C. After the sample had been loaded, SDS-polyacrylamide gel electrophoresis was carried out on a 4-12% bis-tris polyacrylamide gel with 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer (1.00 M MOPS/Tris / 69.3 mM SDS, 20.5 mM EDTA, pH 7.7). Protein bands were detected by Coomassie Brilliant Blue or by silver staining. The Coomassie bands were excised and characterized by MALDI mass spectrometry and amino acid sequence analysis.

Example 6: MALDI mass spectrometry. Coomassie-stained proteins were excised from the gel, repeatedly washed with H₂O and H₂O/acetonitrile and digested overnight with trypsin and endoproteinase Lys-C at 37°C. The peptides generated in the supernatant were analyzed by MALDI mass spectrometry. Sample preparation was achieved following the thin film

preparation techniques (13). Briefly, aliquots of 0.3 μ l of a nitrocellulose containing saturated solution of α -cyano-4- hydroxycinnamic acid in acetone were deposited onto individual spots on the target. Subsequently, 0.8 μ l 10% formic acid and 0.4 μ l of the digest sample was loaded on top of the thin film spots and allowed to dry slowly at ambient temperature. To remove salts from the digestion buffer the spots were washed with 10% formic acid and with H₂O. MALDI mass spectra were recorded in the positive ion mode with delayed extraction on a Reflex II time-of-flight instrument equipped with a SCOUT multiprobe inlet and a 337 nm nitrogen laser. Ion acceleration voltage was set to 20.0 kV, the reflector voltage was set to 21.5 kV and the first extraction plate was set to 15.4 kV. Mass spectra were obtained by averaging 50-200 individual laser shots. Calibration of the spectra was performed internally by a two-point linear fit using the autolysis products of trypsin at m/z 842.50 and m/z 2211.10.

Example 7: *Amino acid sequence analysis of XT.* The MW 120.000 Coomassie-stained protein was excised from the gel, repeatedly washed with H₂O and H₂O/acetonitrile and digested with trypsin and endoproteinase Lys-C overnight. For HPLC separation the excised gel fragment was extracted twice with 0.1% TFA/60% acetonitrile. The extracted enzymatic fragments were separated on a capillary HPLC system equipped with a 140B solvents delivery system (PE Biosystems), Accurate splitter (LC-Packings), UV absorbance detector 759A (PE Biosystems), U-Z capillary flow cell (LC-Packings) and Probot fraction collector (BAI) using reversed-phase column (Hypersil C18 BDS, 3 μ m, 0.3 x 150 mm) and a linear gradient from 12% acetonitrile, 0.1% TFA to 64% acetonitrile, 0.08% TFA in 90 min with a flow rate of 4 μ l/min at RT. Peptide elution was monitored at 214 nm and individual fractions from the HPLC separation were analysed by MALDI mass spectrometry. Sequence analysis of separated fragments was performed on a Procise Protein Sequencer 494 cLC using standard programs supplied by PE Biosystems.

Example 8: *Determination of XT activity.* Determination of XT activity is based on the incorporation of [14 C]-D-xylose into the recombinant bikunin according to a previously described method (Brinkmann et al., 1997, J. Biol. Chem., 272, 11171-11175). For analysis of the substrate specificity of the recombinant xylosyltransferases synthetic peptides containing the XT recognition sequence were used as acceptor. The reaction mixture for the assay contained, in a total volume of 100 μ l: 50 μ l of XT solution, 25 mM 4-morpholineethanesulfonic acid (pH 6.5), 25 mM KCl, 5 mM KF, 5 mM MgCl₂, 5 mM MnCl₂, 1.0 μ M UDP-[¹⁴C]-D-xylose, and 1.5 μ M of the synthetic peptides. After incubation for 75 min at 37°C, the reaction mixtures were placed on discs (25 mm diameter) of Immobilon-

AV membrane, which immobilizes even small peptides by covalent links (Pfund & Bourdage, 1990, *Mol. Immunol.* 27, 495-502), and allowed to dry. It was then washed for 10 min with 10% trichloroacetic acid and three times with 5% trichloroacetic acid solution. Incorporated radioactivity was determined by liquid scintillation counting.

- 5 **Example 9:** *Antiserum Preparation.* The synthetic peptide CSRQKELLKRKLEQKEK deduced from the sequenced peptides 2 and 10 of the enzymatically cleaved XT was synthesized, purified by HPLC and used for immunization of rabbits (BioScience, Germany). Polyclonal antiserum was obtained by injection of the above antigen followed **Example 10:**

Preparation of solid-phase antigen. The antigen CSRQKELLKRKLEQKEK was
10 immobilized on POROS 20 EP. After 1.6 mg antigen was dissolved in 1.2 ml of 10 mM phosphate / 0.15 M NaCl, pH 7.4 0.60 ml of 100 mM phosphate / 1.50 M NaCl, pH 7.4 was added. 400 mg POROS 20 EP was suspended in the solution and the suspension was mixed gently on a shaker at RT. At 10-min intervals, five times in total, an additional 0.24 ml of 100 mM phosphate / 1.50 M NaCl, pH 7.4 was added to the suspension. After additional shaking for
15 5 days at RT the suspension was filtered on a sintered glass funnel. The matrix was suspended in 4 ml 0.2 M phosphate / 0.1 M 2-mercaptoethanol, pH 7.4 and mixed on a shaker for 2 h at RT. The matrix was washed in a sintered glass funnel using 20 ml of 10 mM phosphate / 0.15 M NaCl, pH 7.4 and 20 ml of 1.0 M NaCl. After additional washing with 20 ml of 10 mM phosphate, pH 7.4, the matrix was packed in a PEEK column (4.6 x 50 mm).

- 20 **Example 11:** *Antibody purification.* Antiserum was adjusted to 50 mM Tris/HCl, pH 8.0. The solution was clarified by passage through a 0.2 µm filter. 0.4 ml filtrate was applied at 10 ml/min to the antigen column previously equilibrated with buffer B (50 mM Tris/HCl, pH 8.0). After the column was washed with 4.1 ml buffer B and with 12.5 ml buffer B / 0.15 M NaCl, the adsorbed antibody was eluted using 12.4 ml of 50 mM sodium citrate / 0.15 M NaCl,
25 pH 3.0 followed by 3.4 ml of 100 mM sodium citrate / 1.5 M NaCl, pH 3.0. The eluate was collected as 10 ml fractions in tubes containing 2 ml of 0.5 M Tris/HCl, pH 8.0, to immediately neutralize the citric acid.

- Example 12:** *Preparation of immunoaffinity column.* Purified antibody was concentrated to a protein concentration of 0.3 mg/ml using ultrafiltration with YM1 cellulose membranes.
30 The antibody solution was adjusted to 10 mM phosphate / 0.15 M NaCl, pH 7.4. After filtration of the solution through a 0.2 µm filter 100 µl filtrate was applied at 0.2 ml/min to a POROS 20 PA column (2.1 x 30 mm). This step was repeated 17 times. Adsorbed antibody was cross-linked using cross-linking solution (100 mM triethanolamine, pH 8.5). After the column was

washed with 5 ml of buffer C (10 mM phosphate / 0.15 M NaCl, pH 7.4) 2 ml cross-linking solution was applied at 0.5 ml/min onto the cartridge. The procedure was repeated 6 more times, using a total volume of 14 ml cross-linking solution. To block unreacted functional groups on the cross-linking reagents 2 ml of 100 mM monoethanolamine, pH 9.0

- 5 (quenching solution), was loaded onto the cartridge at 0.5 ml/min. The column was washed using 2 ml of buffer C and the cross-linking step was repeated using another 2 ml quenching solution. The immunoaffinity column was cycled between buffer C and 12 mM HCl / 0.15 M NaCl 3 times using a total volume of 12 ml of solution.

- Example 13:** *Immunoaffinity column⁴ purification of XT.* XT-containing fractions eluted from the heparin affinity matrix were desalted using diafiltration with YM1 cellulose membranes and passed through a 0.2 µm filter. 100 µl of this XT-sample was applied to the immunoaffinity column equilibrated with buffer D (20 mM Tris/HCl, pH 8.0) at a flow rate of 1 ml/min. The column was washed with 1.4 ml of buffer D and with 8.5 ml of buffer D / 0.15 M NaCl. The XT activity was eluted with 4.2 ml of 12 mM HCl followed by 1.2 ml of 12 mM
- 15 HCl / 1.5 M NaCl. Alternatively the elution was performed using 100 µl of antigen at 1 mg/ml in buffer D. Fractions (1 ml) were collected into tubes containing 1 ml of 0.1 M Tris/HCl, pH 8.0. The XT activities of the fractions were determined.

- Example 14:** *Aprotinin affinity chromatography.* 200 µl of desalted XT solution from the heparin purification step was applied at 10 ml/min to the aprotinin column previously
- 20 equilibrated with buffer A. After washing the column with 6.6 ml of buffer A the adsorbed protein was eluted stepwise using the same buffer containing 0.3 M NaCl (10.0 ml), 0.54 M NaCl (10.0 ml), 1.00 M NaCl (10.0 ml) and 1.50 M NaCl (2.4 ml). **Example 15:** *Western Blot Analysis.* For western blot analysis, proteins were transferred to polyvinylidene difluoride membrane in a semi-dry instrument (Novex). After transfer nonspecific
- 25 antibody binding sites were blocked with 2% BSA in 0.1 M Tris/HCl, pH 7.2, for 1 h at RT. The membrane was incubated with antiserum in 50 mM phosphate / 0.15 M NaCl, 0.5 ml/l Tween 20, pH 7.4 at 1:1000 dilution for 1 h. Bound antibody was detected using a second anti-rabbit goat immunoglobulin coupled to horse-radish peroxidase at a 1:1000 dilution. The blot was developed using 4-chloro-1-naphthol.

- 30 **Example 16:** *Gel filtration chromatography.* A sample of 100 µl from the heparin purification step was applied at 1.0 ml/min to a TSK G3000 SW column (30 cm x 7.5 mm, 10 µm particle size) which had previously been equilibrated with buffer A / 0.15 M NaCl. Proteins were eluted with the same buffer. Fractions of 200 µl were collected and tested for XT activity.

Column calibration was performed using thyroglobulin (669.000), ferritin (440.000), aldolase (158.000), albumin (67.000), ovalbumin (43.000).

Example 17: *N-glycosidase F digestion.* Aliquots (1 µg) of XT were digested with 3,1 x 10⁻³ units of N-glycosidase F at 37°C for 1 h and 12 h (Table II). The samples were then
5 subjected to SDS-PAGE, and protein bands were detected by silver staining.

Example 18: *Measurement of protein concentration.* Protein concentration was estimated by absorbance at 280 nm assuming E^{1%}_{1 cm} = 10.0 or with the *Bicinchoninic Acid Protein Assay* using bovine serum albumin as a standard.

Example 19: *PCR-based cloning of human⁴ xylosyltransferase.* Degenerate oligonucleotide
10 primers with deoxyinosine substitution were designed based upon the amino acid sequence of peptides obtained after digestion of the isolated human XT with trypsin or Lys-C. The first strand of cDNA was synthesized by the reverse transcription reaction using poly(A)⁺ RNA isolated from the chondrosarcoma cell line SW1353 as template and oligo(dT) as primer. The reverse transcription reaction was performed at 37°C for 2 h using 50 pmol of oligo(dT)
15 primers, 1 µg of poly(A)⁺ RNA, a 0.5 mM concentration of each dNTP, 1x RT buffer and 200 units of RNase H deficient Moloney murine leukemia virus reverse transcriptase (Life Technologies, Eggenstein, Germany) in a final volume of 20 µl. For PCR amplification the reaction mixture contained 4 µl of the reverse transcription reaction solution, 50 pmol of each primer, a 0.25 mM concentration of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5
20 mM MgCl₂ and 2.5 units of hot start *Taq* polymerase (Life Technologies) in a final volume of 50 µl. Amplification with degenerate oligonucleotide primers was carried out by 40 cycles at 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min, followed by a final extension step at 72°C for 15 min. After agarose electrophoresis of the PCR products, DNA fragments were excised, subcloned into the pCR2.1 vector (Invitrogen, Groningen, Netherlands) and sequenced by the
25 dideoxy chain termination method using *Taq* DNA polymerase (Big-dye terminator cycle sequencing kit, Perkin-Elmer, Norwalk, CT, USA) with an automated DNA sequencer (Applied Biosystems, Weiterstadt, Germany). Several clones were sequenced to compensate for misreading by *Taq* polymerase.

Example 20: *Rapid amplification of 5' and 3' cDNA ends (RACE).* For amplification of the 5'
30 and 3' ends of the XT-I and XT-II isoform cDNA RACE experiments were performed using commercially available systems (Clontech, Heidelberg, Germany; Life Technologies) according to the manufacturers' instructions. For 3'-RACE 1 µg placenta poly(A)⁺ mRNA (Clontech) was reverse transcribed with a 3'-CDS primer (Clontech). PCR amplification of the 3' cDNA end of

human XT-I was accomplished according to a touch-down PCR protocol (5 cycles: 94°C for 30 s, 72°C for 3 min, 5 cycles: 94°C for 30 s, 70°C for 45 s, 72°C for 3 min, 25 cycles: 94°C for 30 s, 65°C for 45 s, 72°C for 3 min) with the gene-specific primer GSP1b 5'-GTGGGTATGCAGAAGTGGGGGAAGGGAC-3' and the UPM primer mix (Clontech). An aliquot of the first PCR was subjected to semi-nested PCR (30 cycles) using the primer Con2111 5'-CCCTCCGCAATGCCTACA-3' and the UPM primer mix. The DNA fragments obtained were subcloned into the vector pCR2.1 (Invitrogen) and sequenced. PCR amplification of the 3' cDNA end of the XT-II isoform was carried out with the primers AB10267 5'-ACTGAGGTCACGCAATACAA-3' and UPM using the touch-down PCR protocol. For 5'-RACE 1 µg of placenta poly(A)⁺ mRNA was subjected to a reverse transcription and 5' tailing reaction with the 5'-CDS primer and the SMART oligo (Clontech) according to the manufacturer's protocol. The 5' end of the XT-I cDNA was amplified using the primer GSP_776L 5'-GCCGCACTCAG-GTGATGAAGAAGT-3' and UPM with a touch-down PCR protocol. An aliquot was used as template in a second semi-nested PCR reaction with the primers GSP_503L 5'-ACCA-CCAGGACAAAGGCGATTCTGA-3' and UPM. The 5'-cDNA end of XT-II was obtained by 5'-RACE amplification using the primers AB10315L 5'-AGTCGAACAGTCCAGG-GCCC-3' and UPM mix. A new primer for a 5'-RACE reaction was designed based upon the nucleotide sequence of the largest fragment obtained (2 kbp), and the experiment was repeated with the primer AB5846L 5'-CACGATCTCGCACTTGGGGG-3' as described above. The nucleotide sequences of human XT-I and XT-II cDNA have been submitted to the GenBank/EBI Data Bank with the accession numbers AJ277441 and AJ277442.

Example 21: *Isolation of XT-I cDNA from human brain and chondrocyte cDNA libraries.*

Plasmid-DNA was isolated from a human whole brain cDNA library (Life Technologies) and a human chondrocyte cDNA library (Clontech) and used as template in a PCR-based approach for isolation of the 5' end of the XT-I cDNA. Briefly, GSP_776L and 5'-ADLD 5'-CTATTCGATGATGAAGATACCCACCAAACCC-3' primers were used in a PCR reaction with 1 µg of plasmid DNA as template. The DNA fragments were subjected to agarose gel electrophoresis and 0.7-3 kb long fragments were excised from the gel and used as a template in a semi-nested PCR reaction with GSP_503L and 5'-ADLD primers. DNA fragments were then subcloned into the vector pCR2.1 and sequenced.

Example 22: *Cloning of rat XT-I and XT-II cDNA.* A RT-PCR based approach using primers based upon the nucleotide sequence of human XT-I and XT-II was employed for amplification of XT-I and XT-II cDNA from rat. The first strand of cDNA was synthesized by the reverse

transcription reaction using poly(A)⁺ RNA isolated from the rat liver cell line BRL3A as template and oligo(dT) as primer. DNA fragments obtained after PCR amplification using moderate stringent conditions were subcloned into the vector pCR2.1 and sequenced. The 5' and 3' ends of the XT-I and XT-II cDNA from rat tissue were amplified using the RACE strategy with gene-specific primers as described above. The nucleotide sequences of rat XT-I and XT-II cDNA have been submitted to the GenBank/EBI Data Bank with the accession numbers AJ295748 and AJ295749.

Example 23: *Expression levels of the XT-I and the XT-II isoform in human tissues.*

A Human Multiple Tissue cDNA Panel (Clontech) was used for the analysis of expression levels. Levels of amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, whose transcript is always present in the tissues at an almost constant level, were determined in parallel for quality control. For amplification of the XT-I encoding transcript the primers 128U and 601L were used, whereas primers AB10267U and AB12394L 5'-GGAAGAGCTGGGTGTGGAAT-3' were employed for the XT-II. PCR reactions were carried out by 22-36 cycles at 94°C for 30 s, 60-65°C for 45 s and 72°C for 2 min. Amplification of a transcript was performed using a serial number of cycles to find the conditions for semiquantitative amplification, and aliquots were analyzed by agarose gel electrophoresis.

Example 24: *Transfection and transient expression of XT.* For construction of a eukaryotic expression vector a DNA fragment including the known coding sequence of XT-I cDNA was amplified by PCR using XT_Exp1L 5'-TTTCCCGTTGAGATCCTGCT-3' and XT_Exp3U 5'-ACAGACAGCAACAACGAGAA-3' as primers and placenta first-strand cDNA (Clontech) as template. The 2450 bp fragment obtained was cloned into the vector pcDNA4/HisMax-TOPO (Invitrogen) resulting in the fusion of XT-I to the *Xpress* epitope. The plasmid pCG227-XT-I was then transiently transfected into CHO-K1 cells. The coding region of the XT-II cDNA was amplified by PCR using the primers AB_Exp9U 5'-AAAGGAAGGCAGAGGAAGC-3' and AB_Exp3L 5'-ACCCCTCCACTGT-CTGTAAG-3' and placenta first-strand cDNA as template. The obtained 2440 bp DNA fragment was cloned into the expression vector pcDNA4/HisMax-TOPO resulting in the fusion of XT-II to the aminoterminal *Xpress* epitope. The plasmid termed pCG226-XT-II was transiently transfected into CHO-K1 cells. 2x10⁵ cells precultured for 1 day in a 35 mm diameter cell culture dish were transfected with 2 µg of plasmid DNA and 6 µl of Fugene 6 transfection reagent (Roche, Mannheim, Germany). For determination of transfection efficiency CHO-K1 cells were transfected with 2 µg of the control plasmid pcDNA4/HisMax-TOPO-*lacZ*. 48 h after transfection the cell culture medium was harvested.

Protein G agarose beads (Sigma, Deisenhofen, Germany) and mouse anti-*Xpress* monoclonal antibody (Invitrogen) were added to the cell culture supernatant and incubated at 4°C for 1 h. After centrifugation at 10.000 g for 1 min the absorbed proteins were twice washed with PBS and resuspended in a final volume of 50 µl. XT activity was then assayed in the samples.

- 5 **Example 25:** *Characterization of the reaction products.* For characterization of the reaction product of recombinant XT-I the peptide QEEEGSGGGQK, which is homologous to the amino terminus of bikunin (Brinkmann *et al.*, 1997, l.c.), was used as acceptor in the XT activity assay. After incubation for 75 min at 37°C the enzyme was heat-inactivated by incubation for 15 min at 65°C and the reaction mixture was used for α- and β-xylosidase treatment and
- 10 alkaline β-elimination. For the linkage-specific digestion of the bound [¹⁴C]-D-xylose 4 mU of α-xylosidase (Seikagaku Corporation, Tokyo, Japan) or 4 mU of β-xylosidase (Sigma, Dreieich, Germany) were added to the samples and incubated for 60 min at 37°C. The reaction mixtures were then placed on Immobilon-AV membrane discs and allowed to dry. The discs were washed with trichloroacetic acid as described above and the remaining incorporated
- 15 radioactivity was determined against appropriate controls. The alkaline cleavage of the O-glycosidic linkage between the [¹⁴C]-D-xylose and the β-hydroxyamino acid serine was performed as described elsewhere (Montreuil *et al.*, 1994). Briefly, the reaction mixture was adjusted to pH 10 with diluted NaOH and an equal volume of cold, freshly prepared sodiumborohydride solution (2 M sodiumborohydride in 0.1 M NaOH) and was added. After
- 20 incubation at 45°C for 16 h the cooled solution was neutralized by adding 50% acetic acid and placed on Immobilon-AV membrane discs. After drying the discs were washed with trichloroacetic acid and the remaining radioactivity was measured by liquid scintillation counting.

Patent Claims

1. An isoform of *UDP-D-xylose:proteoglycan core protein β -D-xylosyltransferase*(XT).
- 5 2. A protein comprising an amino acid sequence of the isoform of claim 1 or a portion thereof, having the biological activity of XT.
3. A protein of claim 1 or 2 deriving from human or rat sources (hXT, rXT)
- 10 4. An isolated protein according to claim 3, wherein said hXT has a molecular weight of 120.000 under SDS polyacrylamide gel electrophoresis conditions.
5. A recombinant protein of claim 3, wherein said protein is hXT-I comprising at least 827 amino acids.
- 15 6. A protein according claim 5, having the amino acid sequence as depicted in Fig. 7B.
7. A protein according to claim 3, wherein said protein is hXT-II comprising 865 amino acids and is an isoform of hXT-I.
- 20 8. A protein according to claim 7 exhibiting approximately 55% overall sequence identity to hXT-I
9. A protein according claim 8, having the amino acid sequence as depicted in Fig. 8B.
- 25 10. A DNA sequence coding for a protein of any of the claims 1 – 9.
11. A DNA sequence according to claim 10 comprising the nucleotide sequence coding for hXT-I as depicted in Fig. 7A, or rXT-I as depicted in Fig. 9.
- 30 12. A DNA sequence according to claim 10 comprising the nucleotide sequence coding for hXT-II as depicted in Fig. 8A or rXT-II as depicted in Fig. 10.

- 33 -

13. An expression vector comprising a promotor sequence, a DNA sequence of claim 10 and optionally a signal sequence.
14. An expression host cell comprising a vector of claim 13, said host cell being capable of
5 expressing a protein of any of the claims 1 – 9.
15. An antibody directed against a protein as defined in any of the claims 1- 9.
16. A process for isolating and purifying a protein as defined in claims 1 – 4, characterized by
10 the following steps:
- (i) culturing cells having an increased level of XT, and harvesting the supernatant of said cell culture,
 - (ii) fractionated ammonium sulfate precipitation of the supernatant of step (i),
 - (iii) heparin affinity chromatography of the precipitate of step (ii),
 - 15 (iv) ion exchange chromatography of the step (iii) product,
 - (v) affinity chromatography of the step (iv) product, and
 - (vi) SDS-Polyacrylamide gel electrophoresis of step (v).
17. A pharmaceutical composition comprising a protein of any of the claims 1 – 9 and a
20 pharmacologically acceptable carrier, diluent or excipient.
18. Use of a protein of any of the claims 1 – 9 for the manufacture of a medicament for the treatment of sclerotic diseases and chronic inflammatory joint diseases.
- 25 19. Use of claim 18, wherein said medicament is an inhibitor or antagonist of said protein.
20. Use of a protein of any of the claims 1 – 9 as diagnostic marker.
21. Use of a DNA molecule of any of the claims 10 – 12 as gene marker.
- 30 22. Use of an antibody as defined in claim 15 in an immunological assay for determination of a protein having the biological activity of hXT as diagnostic tool.

Fig. 1

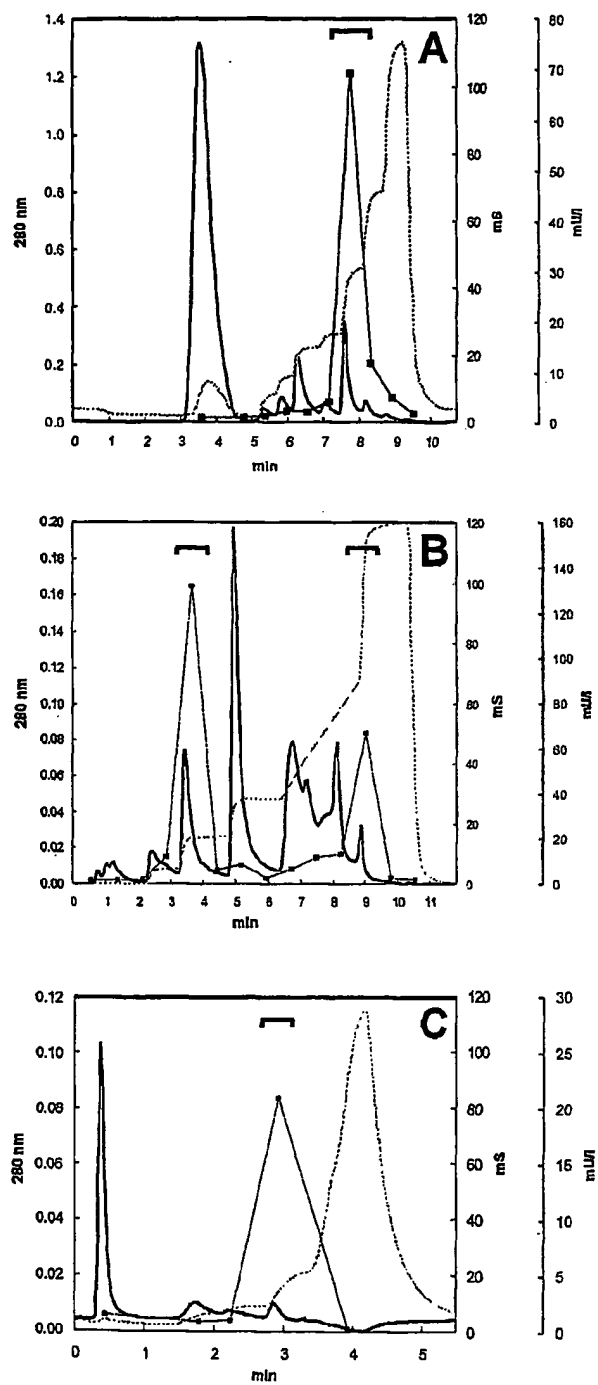


Fig. 2

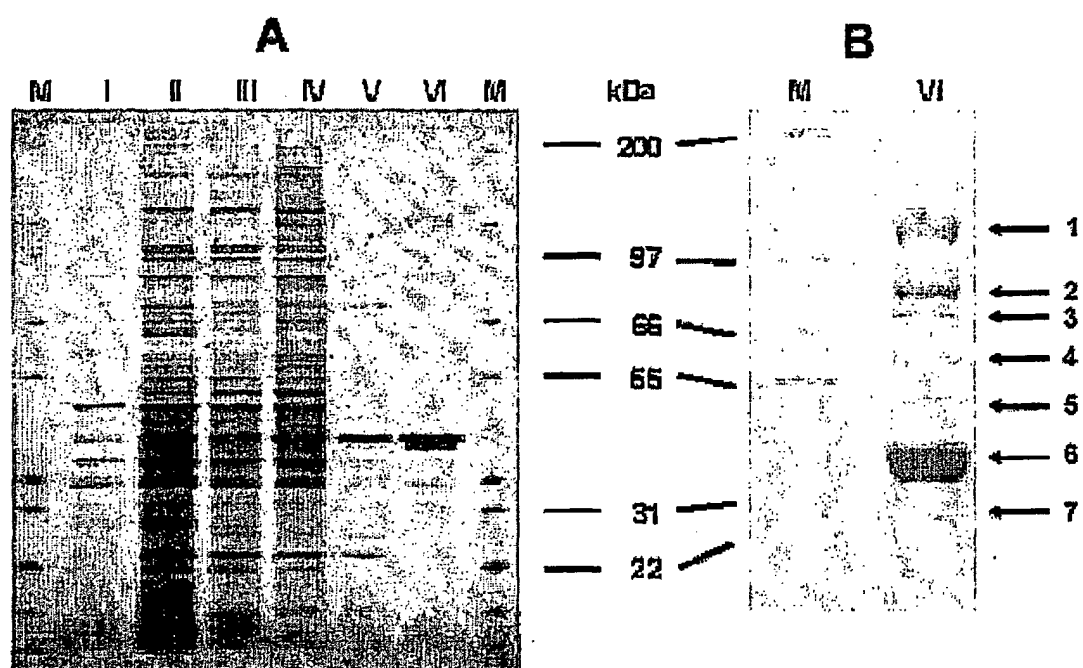


Fig. 3

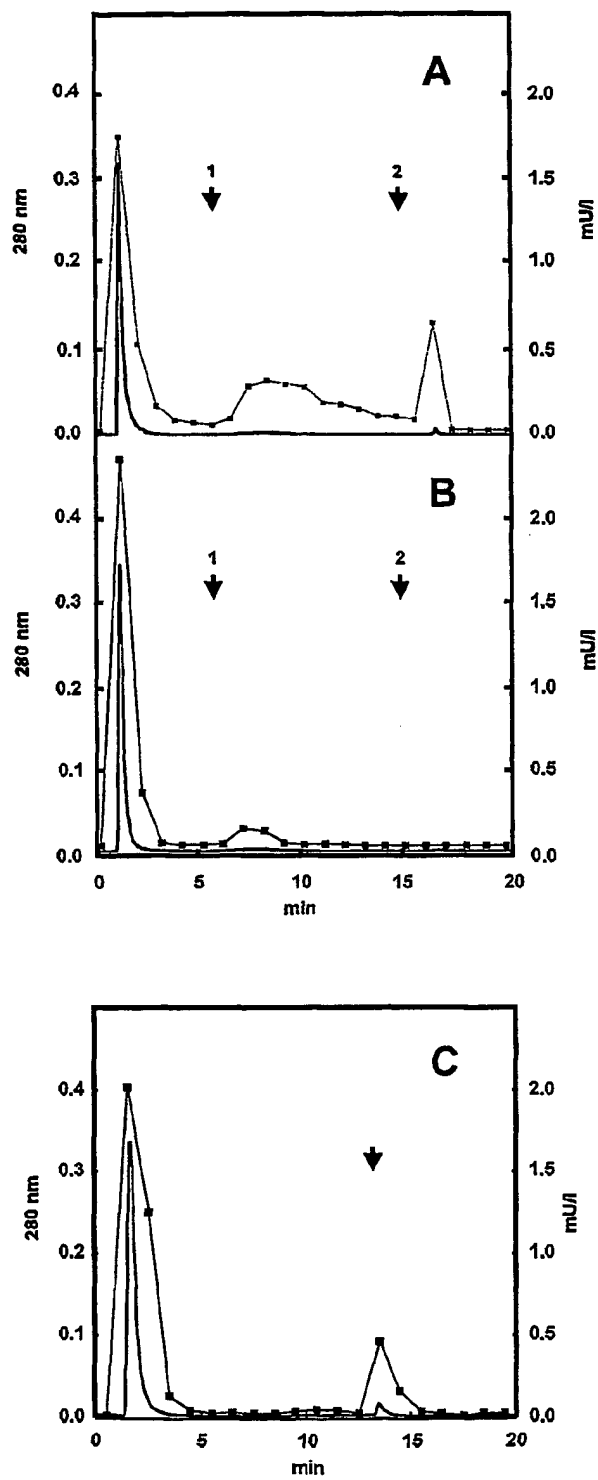
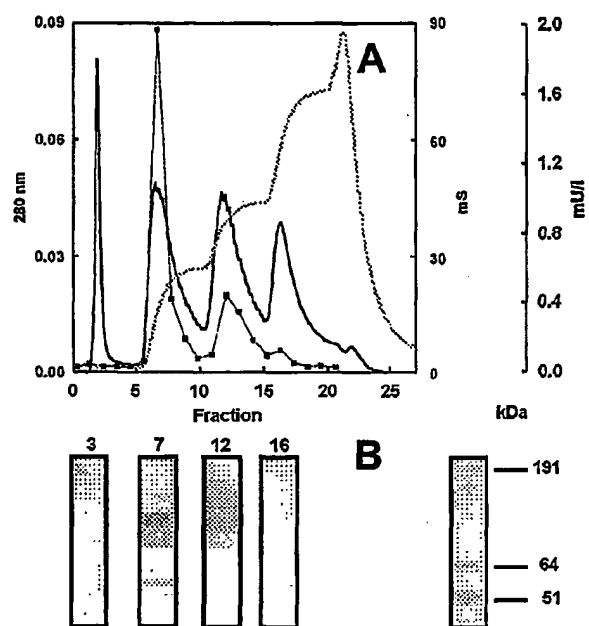
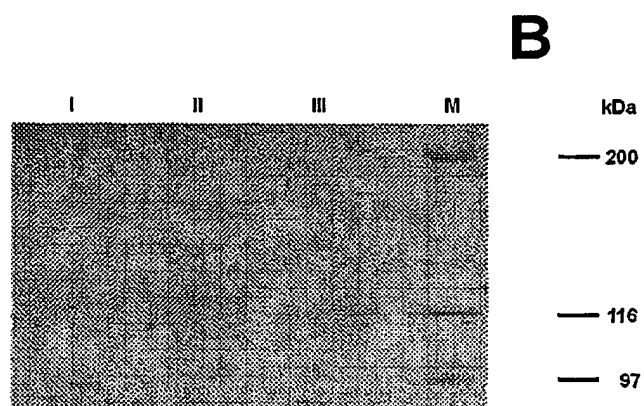
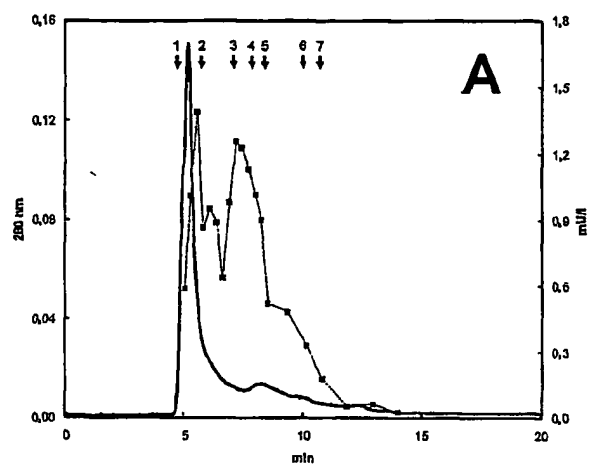


Fig. 4



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Fig. 5



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Fig. 6

(A)

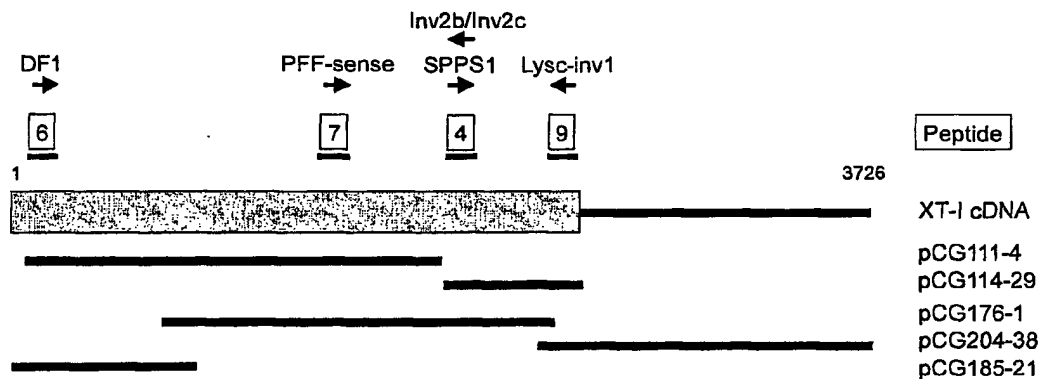
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 Peptide 4 Ile Ala Ser Pro Pro Ser Asp Phe Gly Arg
 Inv2b 5'-TAD CGN AGI GGN GGI AGI CT-3'
 Inv2c 5'-TAD CGN TCR GGI GGI TCR CT-3'

 DF1 5'-GAY TTY GAR AAY GTI GAY AA-3'
 Peptide 6 Asp Phe Glu Asn Val Asp Asn Ser Asn Phe Ala Pro Arg

 PFF-sense 5'-CCI ACI TTY TTY GCN CG-3'
 Peptide 7 Pro Thr Phe Phe Ala Arg

 Peptide 9 Glu Leu Gly Ala Val Lys Pro Asp Gly Arg Leu
 Lysc-inv1 3'-CCN CGI CAN TTY GGN CTR CC-5'

(B)



(C)

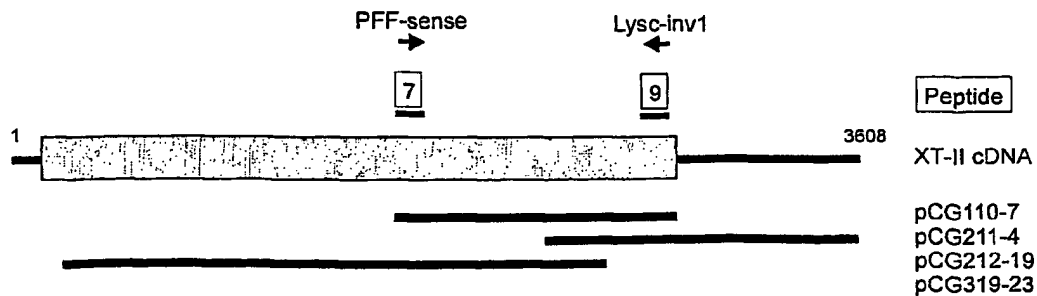


Fig. 7A

ACTCAGGATGGCTACTTTTCTCATCGGCCGAAAGAGAAAGTGCGAACAGACAGCAACAACGAGAACTCT
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TACCTCTTCAGAACCAAGGGCTCTGCAAATGCCAGTTTGACAAGCACTGCCAGACCAACCATGGGTT
CAAACCTCCAGCCCTGCCCTTTGGTTCAATTTTCTGCTTCTTGGCTGGGGGACTCTGGTGCCAGCCTT
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CAAAGTAGCAGAAATAGGATGATGTTTGTGAGCACCAGACTCAAGACCATGACCTTCTTTGATCCTTGA
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CTTGAGACAAAACCTCTGAGCTAGACAGCACAGCAGCCATCCCTGCCAGAGCCCTTCCGGTTTGA
GTCAGACACACAAAACCTTCTGTAATTGCACACCGGTGCTGTTGGGAGTGACCAAAACCATGAACAG
ACTTTTCCCGTCCAGGAAATAGCATTTTCAGATTTGGTTTTTAATTTTCATGCCCTTCGGCCACAGGCTCA
ACGGGACATGCAACATAAAATGGGAAGGTTATTCAACCCGCGGGAAAAA

Fig. 7B

Thr Gln Asp Gly Tyr Phe Ser His Arg Pro Lys Glu Lys Val Arg Thr
 Asp Ser Asn Asn Glu Asn Ser Val Pro Lys Asp Phe Glu Asn Val Asp
 Asn Ser Asn Phe Ala Pro Arg Thr Gln Lys Gln Lys His Gln Pro Glu
 Leu Ala Lys Lys Pro Pro Ser Arg Gln Lys Glu Leu Leu Lys Arg Lys
 Leu Glu Gln Gln Glu Lys Gly Lys Gly His Thr Phe Pro Gly Lys Gly
 Pro Gly Glu Val Leu Pro Pro Gly Asp Arg Ala Ala Ala Asn Ser Ser
 His Gly Lys Asp Val Ser Arg Pro Pro His Ala Arg Lys Thr Gly Gly
 Ser Ser Pro Glu Thr Lys Tyr Asp Gln Pro Pro Lys Cys Asp Ile Ser
 Gly Lys Glu Ala Ile Ser Ala Leu Ser Arg Ala Lys Ser Lys His Cys
 Arg Gln Glu Ile Gly Glu Thr Tyr Cys Arg His Lys Leu Gly Leu Leu
Met Pro Glu Lys Val Thr Arg Phe Cys Pro Leu Glu Gly Lys Ala Asn
 Lys Asn Val Gln Trp Asp Glu Asp Ser Val Glu Tyr Met Pro Ala Asn
 Pro Val Arg Ile Ala Phe Val Leu Val Val His Gly Arg Ala Ser Arg
 Gln Leu Gln Arg Met Phe Lys Ala Ile Tyr His Lys Asp His Phe Tyr
 Tyr Ile His Val Asp Lys Arg Ser Asn Tyr Leu His Arg Gln Val Leu
 Gln Val Ser Arg Gln Tyr Ser Asn Val Arg Val Thr Pro Trp Arg Met
 Ala Thr Ile Trp Gly Gly Ala Ser Leu Leu Ser Thr Tyr Leu Gln Ser
 Met Arg Asp Leu Leu Glu Met Thr Asp Trp Pro Trp Asp Phe Phe Ile
Asn Leu Ser Ala Ala Asp Tyr Pro Ile Arg Thr Asn Asp Gln Leu Val
 Ala Phe Leu Ser Arg Tyr Arg Asp Met Asn Phe Leu Lys Ser His Gly
 Arg Asp Asn Ala Arg Phe Ile Arg Lys Gln Gly Leu Asp Arg Leu Phe
 Leu Glu Cys Asp Ala His Met Trp Arg Leu Gly Asp Arg Arg Ile Pro
 Glu Gly Ile Ala Val Asp Gly Gly Ser Asp Trp Phe Leu Leu Asn Arg
 Arg Phe Val Glu Tyr Val Thr Phe Ser Thr Asp Asp Leu Val Thr Lys
 Met Lys Gln Phe Tyr Ser Tyr Thr Leu Leu Pro Ala Glu Ser Phe Phe
 His Thr Val Leu Glu Asn Ser Pro His Cys Asp Thr Met Val Asp Asn
 Asn Leu Arg Ile Thr Asn Trp Asn Arg Lys Leu Gly Cys Lys Cys Gln
 Tyr Lys His Ile Val Asp Trp Cys Gly Cys Ser Pro Asn Asp Phe Lys
 Pro Gln Asp Phe His Arg Phe Gln Gln Thr Ala Arg Pro Thr Phe Phe
Ala Arg Lys Phe Glu Ala Val Val Asn Gln Glu Ile Ile Gly Gln Leu
 Asp Tyr Tyr Leu Tyr Gly Asn Tyr Pro Ala Gly Thr Pro Gly Leu Arg
 Ser Tyr Trp Glu Asn Val Tyr Asp Glu Pro Asp Gly Ile His Ser Leu
 Ser Asp Val Thr Leu Thr Leu Tyr His Ser Phe Ala Arg Leu Gly Leu
 Arg Arg Ala Glu Thr Ser Leu His Thr Asp Gly Glu Asn Ser Cys Arg
 Tyr Tyr Pro Met Gly His Pro Ala Ser Val His Leu Tyr Phe Leu Ala
 Asp Arg Phe Gln Gly Phe Leu Ile Lys His His Ala Thr Asn Leu Ala
 Val Ser Lys Leu Glu Thr Leu Glu Thr Trp Val Met Pro Lys Lys Val
 Phe Lys Ile Ala Ser Pro Pro Ser Asp Phe Gly Arg Leu Gln Phe Ser
Glu Val Gly Thr Asp Trp Asp Ala Lys Glu Arg Leu Phe Arg Asn Phe
 Gly Gly Leu Leu Gly Pro Met Asp Glu Pro Val Gly Met Gln Lys Trp
 Gly Lys Gly Pro Asn Val Thr Val Thr Val Ile Trp Val Asp Pro Val
 Asn Val Ile Ala Ala Thr Tyr Asp Ile Leu Ile Glu Ser Thr Ala Glu
 Phe Thr His Tyr Lys Pro Pro Leu Asn Leu Pro Leu Arg Pro Gly Val
 Trp Thr Val Lys Ile Leu His His Trp Val Pro Val Ala Glu Thr Lys
 Phe Leu Val Ala Pro Leu Thr Phe Ser Asn Arg Gln Pro Ile Lys Pro
 Glu Glu Ala Leu Lys Leu His Asn Gly Pro Leu Arg Asn Ala Tyr Met
 Glu Gln Ser Phe Gln Ser Leu Asn Pro Val Leu Ser Leu Pro Ile Asn
 Pro Ala Gln Val Glu Gln Ala Arg Arg Asn Ala Ala Ser Thr Gly Thr
 Ala Leu Glu Gly Trp Leu Asp Ser Leu Val Gly Gly Met Trp Thr Ala
 Met Asp Ile Cys Ala Thr Gly Pro Thr Ala Cys Pro Val Met Gln Thr
 Cys Ser Gln Thr Ala Trp Ser Ser Phe Ser Pro Asp Pro Lys Ser Glu
Leu Gly Ala Val Lys Pro Asp Gly Arg Leu Arg

Fig.8A

CGCAGGCCCCGCCCCGCCCCGCCCCGCGCGCGCCCCGCCCCGCTCCCTAGGCGTGGAGGAGGGGGG
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CGAGAAAGGAAGGCAGAGGAAGCCACGGCCACTGGACCCTGGCGAAGGTTCCAAGGACACAGACAGTTCA
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AGTGGCAGATCGTGGGCAAGGACGCACTGTCTGCACTGGCCCCGGCCAGCACCAAGCAGTGCCAGCAGGA
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CTGACTGGGAAGATGAGCCCCGGCATCCAGTGGGATGAGAGCCAAGCCAGCAGCCCATGGATGGCCCCC
CGGTGCGAATCGCCTACATGCTGGTGGTTACGGCCGCGCCATCCGCCAGCTGAAGCGTCTCCCAAGGC
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GAAATATGCAACAGAAGAAATATATCTCTATCTCTCTA

Fig. 8B

Met Val Ala Ser Ala Arg Val Gln Lys Leu Val Arg Arg Tyr Lys Leu Ala
Ile Ala Thr Ala Leu Ala Ile Leu Leu Leu Gln Gly Leu Val Val Trp Ser
 Phe Ser Gly Leu Glu Glu Asp Glu Ala Gly Glu Lys Gly Arg Gln Arg Lys
 Pro Arg Pro Leu Asp Pro Gly Glu Gly Ser Lys Asp Thr Asp Ser Ser Ala
 Gly Arg Arg Gly Ser Thr Gly Arg Arg His Gly Arg Trp Arg Gly Arg Ala
 Glu Ser Pro Gly Val Pro Val Ala Lys Val Val Arg Ala Val Thr Ser Arg
 Gln Arg Ala Ser Arg Arg Val Pro Pro Ala Pro Pro Pro Glu Ala Pro Gly
 Arg Gln Asn Leu Ser Gly Ala Ala Ala Gly Glu Ala Leu Val Gly Ala Ala
 Gly Phe Pro Pro His Gly Asp Thr Gly Ser Val Glu Gly Ala Pro Gln Pro
 Thr Asp Asn Gly Phe Thr Pro Lys Cys Glu Ile Val Gly Lys Asp Ala Leu
 Ser Ala Leu Ala Arg Ala Ser Thr Lys Gln Cys Gln Gln Glu Ile Ala Asn
 Val Val Cys Leu His Gln Ala Gly Ser Leu Met Pro Lys Ala Val Pro Arg
 His Cys Gln Leu Thr Gly Lys Met Ser Pro Gly Ile Gln Trp Asp Glu Ser
 Gln Ala Gln Gln Pro Met Asp Gly Pro Pro Val Arg Ile Ala Tyr Met Leu
 Val Val His Gly Arg Ala Ile Arg Gln Leu Lys Arg Leu Leu Lys Ala Val
 Tyr His Glu Gln His Phe Phe Tyr Ile His Val Asp Lys Arg Ser Asp Tyr
 Leu His Arg Glu Val Val Glu Leu Ala Gln Gly Tyr Asp Asn Val Arg Val
 Thr Pro Trp Arg Met Val Thr Ile Trp Gly Gly Ala Ser Leu Leu Thr Met
 Tyr Leu Arg Ser Met Arg Asp Leu Leu Glu Val Pro Gly Trp Ala Trp Asp
 Phe Phe Ile Asn Leu Ser Ala Thr Asp Tyr Pro Thr Arg Thr Asn Glu Glu
 Leu Val Ala Phe Leu Ser Lys Asn Arg Asp Lys Asn Phe Leu Lys Ser His
 Gly Arg Asp Asn Ser Arg Phe Ile Lys Lys Gln Gly Leu Asp Arg Leu Phe
 His Glu Cys Asp Ser His Met Trp Arg Leu Gly Glu Arg Gln Ile Pro Ala
 Gly Ile Val Val Asp Gly Gly Ser Asp Trp Phe Val Leu Thr Arg Ser Phe
 Val Glu Tyr Val Val Tyr Thr Asp Asp Pro Leu Val Ala Gln Leu Arg Gln
 Phe Tyr Thr Tyr Thr Leu Leu Pro Ala Glu Ser Phe Phe His Thr Val Leu
 Glu Asn Ser Leu Ala Cys Glu Thr Leu Val Asp Asn Asn Leu Arg Val Thr
 Asn Trp Asn Arg Lys Leu Gly Cys Lys Cys Gln Tyr Lys His Ile Val Asp
 Trp Cys Gly Cys Ser Pro Asn Asp Phe Lys Pro Gln Asp Phe Leu Arg Leu
 Gln Gln Val Ser Arg Pro Thr Phe Phe Ala Arg Lys Phe Glu Ser Thr Val
 Asn Gln Glu Val Leu Glu Ile Leu Asp Phe His Leu Tyr Gly Ser Tyr Pro
 Pro Gly Thr Pro Ala Leu Lys Ala Tyr Trp Glu Asn Thr Tyr Asp Ala Ala
 Asp Gly Pro Ser Gly Leu Ser Asp Val Met Leu Thr Ala Tyr Thr Ala Phe
 Ala Arg Leu Ser Leu His His Ala Ala Thr Ala Ala Pro Pro Met Gly Thr
 Pro Leu Cys Arg Phe Glu Pro Arg Gly Leu Pro Ser Ser Val His Leu Tyr
 Phe Tyr Asp Asp His Phe Gln Gly Tyr Leu Val Thr Gln Ala Val Gln Pro
 Ser Ala Gln Gly Pro Ala Glu Thr Leu Glu Met Trp Leu Met Pro Gln Gly
 Ser Leu Lys Leu Leu Gly Arg Ser Asp Gln Ala Ser Arg Leu Gln Ser Leu
 Glu Val Gly Thr Asp Trp Asp Pro Lys Glu Arg Leu Phe Arg Asn Phe Gly
 Gly Leu Leu Gly Pro Leu Asp Glu Pro Val Ala Val Gln Arg Trp Ala Arg
 Gly Pro Asn Leu Thr Ala Thr Val Val Trp Ile Asp Pro Thr Tyr Val Val
 Ala Thr Ser Tyr Asp Ile Thr Val Asp Thr Glu Thr Glu Val Thr Gln Tyr
 Lys Pro Pro Leu Ser Arg Pro Leu Arg Pro Gly Pro Trp Thr Val Arg Leu
 Leu Gln Phe Trp Glu Pro Leu Gly Glu Thr Arg Phe Leu Val Leu Pro Leu
 Thr Phe Asn Arg Lys Leu Pro Leu Arg Lys Asp Asp Ala Ser Trp Leu His
 Ala Gly Pro Pro His Asn Glu Tyr Met Glu Gln Ser Phe Gln Gly Leu Ser
 Ser Ile Leu Asn Leu Pro Gln Pro Glu Leu Ala Glu Glu Ala Ala Gln Arg
 His Thr Gln Leu Thr Gly Pro Ala Leu Glu Ala Trp Thr Asp Arg Glu Leu
 Ser Ser Phe Trp Ser Val Ala Gly Leu Cys Ala Ile Gly Pro Ser Pro Cys
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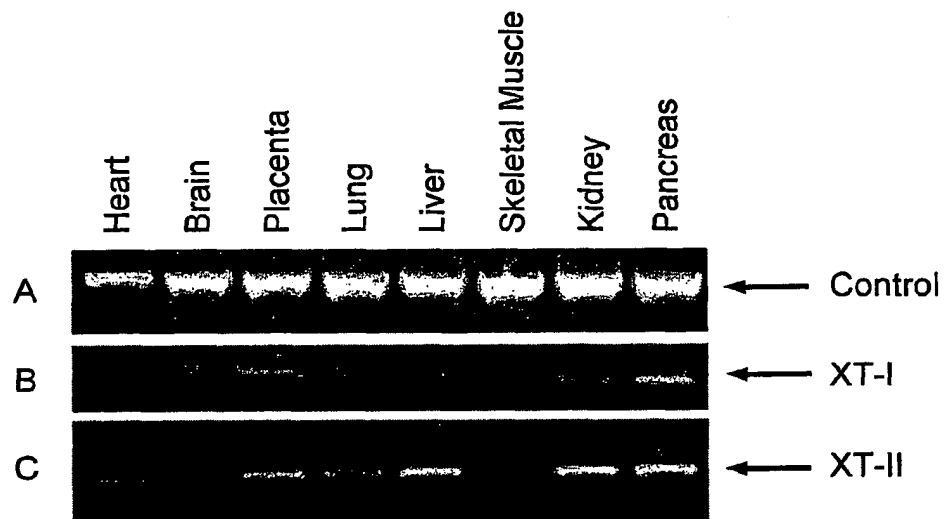
Fig. 9

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Fig. 10

GCGCTCGCGGCCGGGTTCAGGGCCGGACGCGCGCCCCGCGTCCCGGGCAGGAAGATGGTGGCGA
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Fig. 11



SEQUENCE LISTING

<110> Kleesiek, Knut

<120> Xylosyltransferase and isoforms thereof

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Asp	Ser	Asn	Asn	Glu	Asn	Ser	Val	Pro	Lys	Asp	Phe	Glu	Asn	Val	Asp	
		20						25					30			

aac	agc	aac	ttc	gca	ccc	agg	act	caa	aag	cag	aag	cac	cag	cct	gag	144
Asn	Ser	Asn	Phe	Ala	Pro	Arg	Thr	Gln	Lys	Gln	Lys	His	Gln	Pro	Glu	
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Leu	Ala	Lys	Lys	Pro	Pro	Ser	Arg	Gln	Lys	Glu	Leu	Leu	Lys	Arg	Lys	
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gcc gcc act gct gca ccc cca atg ggc acc cca ctc tgc agg ttt gag Ala Ala Thr Ala Ala Pro Pro Met Gly Thr Pro Leu Cys Arg Phe Glu 570 575 580	1901
ccc agg ggc ttg ccg tcc agc gtg cac ctg tat ttc tat gac gac cat Pro Arg Gly Leu Pro Ser Ser Val His Leu Tyr Phe Tyr Asp Asp His 585 590 595 600	1949
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ccg gca gag acg ctt gag atg tgg ctg atg ccc caa ggg tcg ctg aag Pro Ala Glu Thr Leu Glu Met Trp Leu Met Pro Gln Gly Ser Leu Lys 620 625 630	2045
ctg ttg ggg cgc agt gac cag gcc agc cgg ctc cag agt ctg gag gtt Leu Leu Gly Arg Ser Asp Gln Ala Ser Arg Leu Gln Ser Leu Glu Val 635 640 645	2093

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ggc act gat tgg gac ccc aaa gag cgt ctt ttc cgg aac ttt ggg ggg 2141
Gly Thr Asp Trp Asp Pro Lys Glu Arg Leu Phe Arg Asn Phe Gly Gly
650 655 660

tta ctg ggg ccg ctg gac gag cct gtg gcc gtg cag cgc tgg gcc cgg 2189
Leu Leu Gly Pro Leu Asp Glu Pro Val Ala Val Gln Arg Trp Ala Arg
665 670 675 680

ggc ccc aac ctc aca gcc aca gtg gtc tgg atc gac cca acc tat gtg 2237
Gly Pro Asn Leu Thr Ala Thr Val Val Trp Ile Asp Pro Thr Tyr Val
685 690 695

gtg gcc aca tct tat gac atc aca gta gat acg gag act gag gtc acg 2285
Val Ala Thr Ser Tyr Asp Ile Thr Val Asp Thr Glu Thr Glu Val Thr
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caa tac aag ccc cca ctg agc cgg ccc ctg cgg cca ggg ccc tgg act 2333
Gln Tyr Lys Pro Pro Leu Ser Arg Pro Leu Arg Pro Gly Pro Trp Thr
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gtt cga ctc ctt cag ttc tgg gaa ccg ctg ggt gag acc cgc ttc ctt 2381
Val Arg Leu Leu Gln Phe Trp Glu Pro Leu Gly Glu Thr Arg Phe Leu
730 735 740

gtg ctg ccc ttg acc ttc aac cgc aaa cta cct ctc agg aaa gat gat 2429
Val Leu Pro Leu Thr Phe Asn Arg Lys Leu Pro Leu Arg Lys Asp Asp
745 750 755 760

gcc agc tgg ctg cac gca ggg cca ccc cac aac gag tac atg gag cag 2477
Ala Ser Trp Leu His Ala Gly Pro Pro His Asn Glu Tyr Met Glu Gln
765 770 775

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Ser Phe Gln Gly Leu Ser Ser Ile Leu Asn Leu Pro Gln Pro Glu Leu
780 785 790

gcg gag gag gct gcc cag cgg cac aca cag ctc aca ggc cct gcg ctc 2573
Ala Glu Glu Ala Ala Gln Arg His Thr Gln Leu Thr Gly Pro Ala Leu
795 800 805

gag gcc tgg aca gac agg gaa ctg agc agc ttc tgg tcc gtg gct gga 2621
Glu Ala Trp Thr Asp Arg Glu Leu Ser Ser Phe Trp Ser Val Ala Gly
810 815 820

ctg tgt gcc ata ggc ccc tot ccc tgc ccc tcc ctg gag ccc tgc aga 2669
Leu Cys Ala Ile Gly Pro Ser Pro Cys Pro Ser Leu Glu Pro Cys Arg
825 830 835 840

ctg acc agc tgg agc tct ctg tcc ccc gac ccc aaa tca gag ctg ggg 2717
Leu Thr Ser Trp Ser Ser Leu Ser Pro Asp Pro Lys Ser Glu Leu Gly
845 850 855

cct gtc aaa gca gac ggg cga ctc agg tagcagggcc ccagccagta 2764
Pro Val Lys Ala Asp Gly Arg Leu Arg
860 865

cccgtaggagg acccgggaaa ttgcacctta cagacagtgg aggggtgtcc cctcccacag 2824
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<213> Homo sapiens

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      20              25              30

Trp Ser Phe Ser Gly Leu Glu Glu Asp Glu Ala Gly Glu Lys Gly Arg
      35              40              45

Gln Arg Lys Pro Arg Pro Leu Asp Pro Gly Glu Gly Ser Lys Asp Thr
      50              55              60

Asp Ser Ser Ala Gly Arg Arg Gly Ser Thr Gly Arg Arg His Gly Arg
      65              70              75              80

Trp Arg Gly Arg Ala Glu Ser Pro Gly Val Pro Val Ala Lys Val Val
      85              90              95

Arg Ala Val Thr Ser Arg Gln Arg Ala Ser Arg Arg Val Pro Pro Ala
      100             105             110

Pro Pro Pro Glu Ala Pro Gly Arg Gln Asn Leu Ser Gly Ala Ala Ala
      115             120             125

Gly Glu Ala Leu Val Gly Ala Ala Gly Phe Pro Pro His Gly Asp Thr
      130             135             140

Gly Ser Val Glu Gly Ala Pro Gln Pro Thr Asp Asn Gly Phe Thr Pro
      145             150             155             160

Lys Cys Glu Ile Val Gly Lys Asp Ala Leu Ser Ala Leu Ala Arg Ala
      165             170             175

Ser Thr Lys Gln Cys Gln Gln Glu Ile Ala Asn Val Val Cys Leu His
      180             185             190

Gln Ala Gly Ser Leu Met Pro Lys Ala Val Pro Arg His Cys Gln Leu
      195             200             205

Thr Gly Lys Met Ser Pro Gly Ile Gln Trp Asp Glu Ser Gln Ala Gln
      210             215             220

Gln Pro Met Asp Gly Pro Pro Val Arg Ile Ala Tyr Met Leu Val Val
      225             230             235             240

His Gly Arg Ala Ile Arg Gln Leu Lys Arg Leu Leu Lys Ala Val Tyr
      245             250             255

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His Glu Gln His Phe Phe Tyr Ile His Val Asp Lys Arg Ser Asp Tyr
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 Leu His Arg Glu Val Val Glu Leu Ala Gln Gly Tyr Asp Asn Val Arg
 275 280 285
 Val Thr Pro Trp Arg Met Val Thr Ile Trp Gly Gly Ala Ser Leu Leu
 290 295 300
 Thr Met Tyr Leu Arg Ser Met Arg Asp Leu Leu Glu Val Pro Gly Trp
 305 310 315 320
 Ala Trp Asp Phe Phe Ile Asn Leu Ser Ala Thr Asp Tyr Pro Thr Arg
 325 330 335
 Thr Asn Glu Glu Leu Val Ala Phe Leu Ser Lys Asn Arg Asp Lys Asn
 340 345 350
 Phe Leu Lys Ser His Gly Arg Asp Asn Ser Arg Phe Ile Lys Lys Gln
 355 360 365
 Gly Leu Asp Arg Leu Phe His Glu Cys Asp Ser His Met Trp Arg Leu
 370 375 380
 Gly Glu Arg Gln Ile Pro Ala Gly Ile Val Val Asp Gly Gly Ser Asp
 385 390 395 400
 Trp Phe Val Leu Thr Arg Ser Phe Val Glu Tyr Val Val Tyr Thr Asp
 405 410 415
 Asp Pro Leu Val Ala Gln Leu Arg Gln Phe Tyr Thr Tyr Thr Leu Leu
 420 425 430
 Pro Ala Glu Ser Phe Phe His Thr Val Leu Glu Asn Ser Leu Ala Cys
 435 440 445
 Glu Thr Leu Val Asp Asn Asn Leu Arg Val Thr Asn Trp Asn Arg Lys
 450 455 460
 Leu Gly Cys Lys Cys Gln Tyr Lys His Ile Val Asp Trp Cys Gly Cys
 465 470 475 480
 Ser Pro Asn Asp Phe Lys Pro Gln Asp Phe Leu Arg Leu Gln Gln Val
 485 490 495
 Ser Arg Pro Thr Phe Phe Ala Arg Lys Phe Glu Ser Thr Val Asn Gln
 500 505 510
 Glu Val Leu Glu Ile Leu Asp Phe His Leu Tyr Gly Ser Tyr Pro Pro
 515 520 525
 Gly Thr Pro Ala Leu Lys Ala Tyr Trp Glu Asn Thr Tyr Asp Ala Ala
 530 535 540
 Asp Gly Pro Ser Gly Leu Ser Asp Val Met Leu Thr Ala Tyr Thr Ala
 545 550 555 560
 Phe Ala Arg Leu Ser Leu His His Ala Ala Thr Ala Ala Pro Pro Met
 565 570 575
 Gly Thr Pro Leu Cys Arg Phe Glu Pro Arg Gly Leu Pro Ser Ser Val
 580 585 590

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His Leu Tyr Phe Tyr Asp Asp His Phe Gln Gly Tyr Leu Val Thr Gln
 595 600 605
 Ala Val Gln Pro Ser Ala Gln Gly Pro Ala Glu Thr Leu Glu Met Trp
 610 615 620
 Leu Met Pro Gln Gly Ser Leu Lys Leu Leu Gly Arg Ser Asp Gln Ala
 625 630 635 640
 Ser Arg Leu Gln Ser Leu Glu Val Gly Thr Asp Trp Asp Pro Lys Glu
 645 650 655
 Arg Leu Phe Arg Asn Phe Gly Gly Leu Leu Gly Pro Leu Asp Glu Pro
 660 665 670
 Val Ala Val Gln Arg Trp Ala Arg Gly Pro Asn Leu Thr Ala Thr Val
 675 680 685
 Val Trp Ile Asp Pro Thr Tyr Val Val Ala Thr Ser Tyr Asp Ile Thr
 690 695 700
 Val Asp Thr Glu Thr Glu Val Thr Gln Tyr Lys Pro Pro Leu Ser Arg
 705 710 715 720
 Pro Leu Arg Pro Gly Pro Trp Thr Val Arg Leu Leu Gln Phe Trp Glu
 725 730 735
 Pro Leu Gly Glu Thr Arg Phe Leu Val Leu Pro Leu Thr Phe Asn Arg
 740 745 750
 Lys Leu Pro Leu Arg Lys Asp Asp Ala Ser Trp Leu His Ala Gly Pro
 755 760 765
 Pro His Asn Glu Tyr Met Glu Gln Ser Phe Gln Gly Leu Ser Ser Ile
 770 775 780
 Leu Asn Leu Pro Gln Pro Glu Leu Ala Glu Glu Ala Ala Gln Arg His
 785 790 795 800
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 Ser Ser Phe Trp Ser Val Ala Gly Leu Cys Ala Ile Gly Pro Ser Pro
 820 825 830
 Cys Pro Ser Leu Glu Pro Cys Arg Leu Thr Ser Trp Ser Ser Leu Ser
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gag aac tca gtc ccc aag gac ttt gag aat gtg gac aac agc aac ttc	96
Glu Asn Ser Val Pro Lys Asp Phe Glu Asn Val Asp Asn Ser Asn Phe	
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gca ccc agg act caa aag cag aag cac cag cca gag ttg gca aag aag	144
Ala Pro Arg Thr Gln Lys Gln Lys His Gln Pro Glu Leu Ala Lys Lys	
35 40 45	
ccc ctc agc agg cag aaa gag cgt ttg cag aga aag ctg ggc gcc cag	192
Pro Leu Ser Arg Gln Lys Glu Arg Leu Gln Arg Lys Leu Gly Ala Gln	
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gac aaa gga cag ggg cag tca gtc cta gga aaa ggc ccc aag gag gtg	240
Asp Lys Gly Gln Gly Gln Ser Val Leu Gly Lys Gly Pro Lys Glu Val	
65 70 75 80	
ctg cct cct cgg gaa aaa gcc cca ggc aac agt agc caa ggg aag gat	288
Leu Pro Pro Arg Glu Lys Ala Pro Gly Asn Ser Ser Gln Gly Lys Asp	
85 90 95	
ctc tca aga cac agc cat agc agg aag agt ggt ggc ggt ggg tcc ccg	336
Leu Ser Arg His Ser His Ser Arg Lys Ser Gly Gly Gly Gly Ser Pro	
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gaa act aag tct gac cag gtc ccc aag tgt gac atc tct ggc aag gag	384
Glu Thr Lys Ser Asp Gln Val Pro Lys Cys Asp Ile Ser Gly Lys Glu	
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gcc atc tca gcg ctg acc cgc gct aag tcc aag cac tgt cgc cag gag	432
Ala Ile Ser Ala Leu Thr Arg Ala Lys Ser Lys His Cys Arg Gln Glu	
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Ile Ala Glu Thr Tyr Cys Arg His Lys Leu Gly Leu Leu Met Pro Glu	
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Lys Val Ala Arg Phe Cys Pro Leu Glu Gly Lys Ala Asn Lys Asn Val	
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cag tgg gat gag gat gct gtt gag tac atg ccc ccc aac cca gtc aga	576
Gln Trp Asp Glu Asp Ala Val Glu Tyr Met Pro Pro Asn Pro Val Arg	
180 185 190	
atc gcc ttt gtc ctg gtg gtc cat ggc cgt gcc tct cga cag ctg cag	624
Ile Ala Phe Val Leu Val Val His Gly Arg Ala Ser Arg Gln Leu Gln	
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cgc atg ttt aag gcc atc tac cac aaa gac cat ttc tac tat atc cat	672
Arg Met Phe Lys Ala Ile Tyr His Lys Asp His Phe Tyr Tyr Ile His	
210 215 220	
gtg gat aag cgt tcc aat tac ctg cat cgg caa gtg ctc cag ttc tcc	720
Val Asp Lys Arg Ser Asn Tyr Leu His Arg Gln Val Leu Gln Phe Ser	
225 230 235 240	
agg cag tac gac aac gtc cga gtc act tcc tgg agg atg gcc acc att	768
Arg Gln Tyr Asp Asn Val Arg Val Thr Ser Trp Arg Met Ala Thr Ile	
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tgg ggc gga gcc agc ctc ctg tcc act tac ctg cag agc atg cgg gat	816
Trp Gly Gly Ala Ser Leu Leu Ser Thr Tyr Leu Gln Ser Met Arg Asp	
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Leu Leu Glu Met Thr Asp Trp Pro Trp Asp Phe Phe Ile Asn Leu Ser	
275 280 285	
gcg gct gac tac ccc atc agg aca aat gac cag ctc gta gca ttt ctt	912
Ala Ala Asp Tyr Pro Ile Arg Thr Asn Asp Gln Leu Val Ala Phe Leu	
290 295 300	
tcc aga tac cga gat atg aat ttc ctg aag tca cat ggt cgg gac aat	960
Ser Arg Tyr Arg Asp Met Asn Phe Leu Lys Ser His Gly Arg Asp Asn	
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gca agg ttc atc cgg aag cag gac ctg gac cgt ctc ttc ctg gag tgt	1008
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Asp Thr His Met Trp Arg Leu Gly Asp Arg Arg Ile Pro Glu Gly Ile	
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Glu Tyr Val Ala Phe Ser Thr Asp Asp Leu Val Thr Lys Met Lys Gln	
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Phe Tyr Ser Tyr Thr Leu Leu Pro Ala Glu Ser Phe Phe His Thr Val	
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Ile Thr Asn Trp Asn Arg Lys Leu Gly Cys Lys Cys Gln Tyr Lys His	
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ttc cat cgc ttc cag cag acg gcc cgg ccc acc ttc ttt gcc cga aag	1392
Phe His Arg Phe Gln Gln Thr Ala Arg Pro Thr Phe Phe Ala Arg Lys	
450 455 460	
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Phe Glu Ala Ile Val Asn Gln Glu Ile Ile Gly Gln Leu Asp Ser Tyr	
465 470 475 480	
ctg tat ggc aac tat cct gcg ggc acc ccg ggc ctc cgc tct tac tgg	1488
Leu Tyr Gly Asn Tyr Pro Ala Gly Thr Pro Gly Leu Arg Ser Tyr Trp	
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Glu Asn Val Tyr Asp Glu Pro Asp Gly Ile Gln Ser Leu Ser Asp Val	
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Glu Ser Ser Leu His Thr Asp Gly Glu Asn Ser Cys Arg Tyr Tyr Pro	
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Met Gly His Pro Ala Ser Val His Leu Tyr Phe Leu Ala Asp Arg Phe	
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Gln Gly Phe Leu Ile Lys His His Val Thr Asn Leu Ala Val Ser Lys	
565 570 575	
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Leu Gly Pro Met Asp Glu Pro Val Gly Met Gln Lys Trp Gly Lys Gly	
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Pro Asn Val Thr Val Thr Val Ile Trp Val Asp Pro Val Asn Val Ile	
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gca gcc acc tat gat atc ctg atc gag tcc act gcg gaa ttc aca cac	2016
Ala Ala Thr Tyr Asp Ile Leu Ile Glu Ser Thr Ala Glu Phe Thr His	
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Tyr Lys Pro Pro Leu Asn Leu Pro Leu Arg Pro Gly Val Trp Thr Val	
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Lys Ile Leu His His Trp Val Pro Val Ala Glu Thr Lys Phe Leu Val	
690 695 700	
gca cct ctg acc ttc tcc aac aag cag ccc atc aaa cca gag gag gct	2160
Ala Pro Leu Thr Phe Ser Asn Lys Gln Pro Ile Lys Pro Glu Glu Ala	
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Phe Gln Ser Leu Asn Pro Val Leu Ser Leu His Ile Asn Pro Ala Gln	
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gtg gag cag gcc cgg aag aac gca gcc ttc act ggg aca gca cta gaa	2304
Val Glu Gln Ala Arg Lys Asn Ala Ala Phe Thr Gly Thr Ala Leu Glu	
755 760 765	

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 Ala Trp Leu Val Gly Gly Thr Trp Thr Ala Met Asp Val Cys Ala Thr
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ggc ccc act gcc tgc cca gtc atg caa acc tgc agc caa aca gcc tgg 2400
 Gly Pro Thr Ala Cys Pro Val Met Gln Thr Cys Ser Gln Thr Ala Trp
 785 790 795 800

agt tcc ttc agc cct gat ccc aag tca gag ctg ggt gca gtg aag cct 2448
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gat ggt cgg ctc agg 2463
 Asp Gly Arg Leu Arg
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Pro Leu Ser Arg Gln Lys Glu Arg Leu Gln Arg Lys Leu Gly Ala Gln
 50 55 60

Asp Lys Gly Gln Gly Gln Ser Val Leu Gly Lys Gly Pro Lys Glu Val
 65 70 75 80

Leu Pro Pro Arg Glu Lys Ala Pro Gly Asn Ser Ser Gln Gly Lys Asp
 85 90 95

Leu Ser Arg His Ser His Ser Arg Lys Ser Gly Gly Gly Gly Ser Pro
 100 105 110

Glu Thr Lys Ser Asp Gln Val Pro Lys Cys Asp Ile Ser Gly Lys Glu
 115 120 125

Ala Ile Ser Ala Leu Thr Arg Ala Lys Ser Lys His Cys Arg Gln Glu
 130 135 140

Ile Ala Glu Thr Tyr Cys Arg His Lys Leu Gly Leu Leu Met Pro Glu
 145 150 155 160

Lys Val Ala Arg Phe Cys Pro Leu Glu Gly Lys Ala Asn Lys Asn Val
 165 170 175

Gln Trp Asp Glu Asp Ala Val Glu Tyr Met Pro Pro Asn Pro Val Arg
 180 185 190

Ile Ala Phe Val Leu Val Val His Gly Arg Ala Ser Arg Gln Leu Gln
 195 200 205

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Arg Met Phe Lys Ala Ile Tyr His Lys Asp His Phe Tyr Tyr Ile His
 210 215 220
 Val Asp Lys Arg Ser Asn Tyr Leu His Arg Gln Val Leu Gln Phe Ser
 225 230 235 240
 Arg Gln Tyr Asp Asn Val Arg Val Thr Ser Trp Arg Met Ala Thr Ile
 245 250 255
 Trp Gly Gly Ala Ser Leu Leu Ser Thr Tyr Leu Gln Ser Met Arg Asp
 260 265 270
 Leu Leu Glu Met Thr Asp Trp Pro Trp Asp Phe Phe Ile Asn Leu Ser
 275 280 285
 Ala Ala Asp Tyr Pro Ile Arg Thr Asn Asp Gln Leu Val Ala Phe Leu
 290 295 300
 Ser Arg Tyr Arg Asp Met Asn Phe Leu Lys Ser His Gly Arg Asp Asn
 305 310 315 320
 Ala Arg Phe Ile Arg Lys Gln Asp Leu Asp Arg Leu Phe Leu Glu Cys
 325 330 335
 Asp Thr His Met Trp Arg Leu Gly Asp Arg Arg Ile Pro Glu Gly Ile
 340 345 350
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 355 360 365
 Glu Tyr Val Ala Phe Ser Thr Asp Asp Leu Val Thr Lys Met Lys Gln
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 465 470 475 480
 Leu Tyr Gly Asn Tyr Pro Ala Gly Thr Pro Gly Leu Arg Ser Tyr Trp
 485 490 495
 Glu Asn Val Tyr Asp Glu Pro Asp Gly Ile Gln Ser Leu Ser Asp Val
 500 505 510
 Ala Leu Thr Met Tyr His Ser Phe Ile Arg Leu Gly Leu Arg Arg Ala
 515 520 525
 Glu Ser Ser Leu His Thr Asp Gly Glu Asn Ser Cys Arg Tyr Tyr Pro
 530 535 540

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Met Gly His Pro Ala Ser Val His Leu Tyr Phe Leu Ala Asp Arg Phe
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 565 570 575
 Leu Glu Thr Leu Glu Thr Trp Met Met Pro Lys Lys Val Phe Lys Val
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 Tyr Lys Pro Pro Leu Asn Leu Pro Leu Arg Pro Gly Val Trp Thr Val
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<213> Rat coronavirus

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Ala Val Thr Ser Arg Gln Arg Ala Ser Arg Arg Val Pro Pro Ala Pro
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Ser Val Glu Gly Ala Pro Gln Pro Thr Asp Asn Ser Phe Thr Pro Lys
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Thr Lys His Cys Gln Gln Glu Ile Ala Asn Val Val Cys Leu His Gln
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Tyr Arg Glu Val Val Glu Leu Ala Gln His Tyr Asp Asn Val Arg Val	
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Gln Pro Ser Ala Gln Gly Pro Ala Glu Thr Leu Glu Met Trp Leu Met	
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 755 760 765

aac gaa tac atg gaa cag agt ttc cag gga cta agt ggc atc ctg aat 2410
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 790 795 800

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 35 40 45

Gln Arg Lys Pro Arg Pro Leu Asp Pro Gly Glu Gly Ser Lys Asp Thr
 50 55 60

Asp Ser Ser Ala Gly Arg Arg Gly Ser Ala Gly Arg Arg His Gly Arg
 65 70 75 80

Trp Arg Gly Arg Ala Glu Ser Pro Gly Val Pro Val Ala Lys Val Val
 85 90 95

Arg Ala Val Thr Ser Arg Gln Arg Ala Ser Arg Arg Val Pro Pro Ala
 100 105 110

Pro Pro Pro Glu Ala Pro Gly Arg Gln Asn Leu Ser Gly Ala Ala Ala
 115 120 125

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Gly Glu Ala Leu Ile Gly Ala Ala Gly Phe Pro Gln His Gly Asp Thr
 130 135 140

Gly Ser Val Glu Gly Ala Pro Gln Pro Thr Asp Asn Ser Phe Thr Pro
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Lys Cys Glu Ile Val Gly Lys Asp Ala Leu Ser Ala Leu Ala Arg Ala
 165 170 175

Ser Thr Lys His Cys Gln Gln Glu Ile Ala Asn Val Val Cys Leu His
 180 185 190

Gln Ala Gly Asn Leu Met Pro Lys Ser Val Pro Arg His Cys Gln Leu
 195 200 205

Ala Gly Lys Val Ser Pro Gly Ile Gln Trp Glu Glu Val Arg Ala Gln
 210 215 220

Gln Pro Val Ser Gly Pro Leu Val Arg Ile Ala Tyr Met Leu Val Val
 225 230 235 240

His Gly Arg Ala Val Arg Gln Leu Lys Arg Leu Leu Lys Ala Val Tyr
 245 250 255

His Glu Glu His Phe Phe Tyr Ile His Val Asp Lys Arg Ser Asn Tyr
 260 265 270

Leu Tyr Arg Glu Val Val Glu Leu Ala Gln His Tyr Asp Asn Val Arg
 275 280 285

Val Thr Pro Trp Arg Met Val Thr Ile Trp Gly Gly Ala Ser Leu Leu
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Arg Met Tyr Leu Arg Ser Met Lys Asp Leu Leu Glu Thr Pro Gly Trp
 305 310 315 320

Thr Trp Asp Phe Phe Ile Asn Leu Ser Ala Thr Asp Tyr Pro Thr Arg
 325 330 335

Thr Asn Glu Glu Leu Val Ala Phe Leu Ser Lys Asn Arg Asp Lys Asn
 340 345 350

Phe Leu Lys Ser His Gly Arg Asp Asn Ser Arg Phe Ile Lys Lys Gln
 355 360 365

Gly Leu Asp Arg Leu Phe His Glu Cys Asp Ser His Met Trp Arg Leu
 370 375 380

Gly Glu Arg Gln Ile Pro Ala Gly Ile Val Val Asp Gly Gly Ser Asp
 385 390 395 400

Trp Phe Val Leu Thr Arg Ser Phe Val Glu Tyr Val Val Tyr Thr Glu
 405 410 415

Asp Pro Leu Val Ala Gln Leu Arg Gln Phe Tyr Thr Tyr Thr Leu Leu
 420 425 430

Pro Ala Glu Ser Phe Phe His Thr Val Leu Glu Asn Ser Pro Ala Cys
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Glu Ser Leu Val Asp Asn Asn Leu Arg Val Thr Asn Trp Asn Arg Lys
 450 455 460

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Leu Gly Cys Lys Cys Gln Tyr Lys His Ile Val Asp Trp Cys Gly Cys
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Ser Pro Asn Asp Phe Lys Pro Gln Asp Phe Leu Arg Leu Gln Gln Val
 485 490 495

Ser Arg Pro Thr Phe Phe Ala Arg Lys Phe Glu Ser Thr Val Asn Gln
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Glu Val Leu Glu Ile Leu Asp Phe His Leu Tyr Gly Ser Tyr Pro Pro
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Gly Thr Pro Ala Leu Lys Ala Tyr Trp Glu Asn Ile Tyr Asp Met Ala
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Asp Gly Pro Ser Gly Leu Ser Asp Val Leu Leu Thr Ala Tyr Thr Ala
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Phe Ala Arg Ile Ser Leu Arg His Ala Ala Thr Val Ser Pro Leu Ala
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Thr Ala Val Cys Arg Phe Glu Pro Arg Gly Leu Pro Ser Ser Val His
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Val Gln Pro Ser Ala Gln Gly Pro Ala Glu Thr Leu Glu Met Trp Leu
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Met Pro Gln Arg Leu Leu Lys Pro Leu Gly His Ser Asp Gln Ala Ser
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Arg Leu Gln Ser Leu Glu Val Gly Thr Glu Trp Asp Pro Lys Glu Arg
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Leu Phe Arg Asn Phe Gly Gly Leu Leu Gly Pro Leu Asp Glu Pro Val
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Ala Met Gln Arg Trp Ala Arg Gly Pro Asn Leu Thr Ala Thr Val Val
 675 680 685

Trp Ile Asp Pro Thr Tyr Val Val Ala Thr Ser Tyr Asp Ile Thr Val
 690 695 700

Asp Ala Asp Thr Glu Val Thr Gln Tyr Lys Pro Pro Leu Ser Leu Pro
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Leu Pro Leu Arg Lys Asp Asp Ala Ser Trp Leu His Ala Gly Pro Pro
 755 760 765

His Asn Glu Tyr Met Glu Gln Ser Phe Gln Gly Leu Ser Gly Ile Leu
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Asn Leu Pro Gln Pro Glu Ala Val Glu Glu Ala Ala Arg Arg His Thr
 785 790 795 800

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Glu Leu Thr Gly Pro Ala Leu Glu Ala Trp Thr Asp Gly Glu Leu Ser
805 810 815

Ser Phe Trp Ser Val Ala Gly Leu Cys Ala Ile Gly Pro Ser Ser Cys
820 825 830

Pro Ser Leu Glu Leu Cys Arg Leu Thr Ser Trp Ser Ser Leu Ser Pro
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Lys

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<212> PRT

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<210> 12

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<212> PRT

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<223> Description of Artificial Sequence: digestion
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Asp Met Asn Phe Leu Lys

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<210> 29

<211> 10

<212> PRT

<213> Artificial Sequence

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<210> 31

<211> 13

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<223> Description of Artificial Sequence: digestion
peptide

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<211> 6

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<210> 33

<211> 12

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence: digestion
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<210> 34

<211> 11

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence: digestion
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<211> 12

<212> PRT

<213> Artificial Sequence

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<211> 8

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: digestion
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Leu Gly Leu Leu Met Pro Glu Lys
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ning of each regular issue of the PCT Gazette.*

(54) Title: XYLOSYLTRANSFERASE AND ISOFORMS THEREOF

(57) Abstract: The invention relates to the isolation, purification and characterization of the enzyme xylosyltransferase (defined as "XT"). The invention describes for the first time that XT occurs in at least two isoforms ("XT-I", "XT-II"). The invention relates furthermore to the recombinant cloning and expression of human and rat XT-I and XT-II and discloses their DNA and protein sequences. The enzymes according to the invention can be used as therapeutic agents and as diagnostic markers, e.g. for the determination of enhanced proteoglycan biosynthesis, and as biochemical markers for determination of several pathological processes such as systemic sclerosis.

WO 01/49831 A3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/13311

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/10 C12N15/63 C07K1/36 C07K16/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, WPI Data, EPO-Internal, MEDLINE, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GOETTING CHRISTIAN ET AL: "Xylosylation of alternatively spliced isoforms of Alzheimer APP by xylosyltransferase." JOURNAL OF PROTEIN CHEMISTRY, vol. 17, no. 3, April 1998 (1998-04), pages 295-302, XP000996086 ISSN: 0277-8033 the whole document --- -/--	1-6,10, 11,13-22



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

2 August 2001

Date of mailing of the international search report

20. 08. 01

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Schwachtgen, J-L

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/13311

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>GOETTING CHRISTIAN ET AL: "Serum xylosyltransferase: A new biochemical marker of the sclerotic process in systemic sclerosis." JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 112, no. 6, June 1999 (1999-06), pages 919-924, XP002165496 ISSN: 0022-202X cited in the application the whole document</p>	1-6
P,X	<p>GOETTING CHRISTIAN ET AL: "Molecular cloning and expression of human UDP-D-xylose:proteoglycan core protein beta-D-xylosyltransferase and its first isoform XT-II." JOURNAL OF MOLECULAR BIOLOGY, vol. 304, no. 4, 8 December 2000 (2000-12-08), pages 517-528, XP002165497 ISSN: 0022-2836 the whole document</p>	1-22
X	<p>DATABASE EMBL 'Online!' EBI; Accession No: AC004707, 21 May 1998 (1998-05-21) BIRREN B. ET AL.: "Homo sapiens chromosome 17, clone hRPC.117_B_12, complete sequence." XP002173828 abstract</p>	10,12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 00/13311

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 20-22 are directed to a diagnostic method practised on the human body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 4-6, 11 and 1-3, 10, 13-22 (partially)

UDP-D-xylose proteoglycan core protein
beta-D-xylosyltransferase I (XT-I) and its use

2. Claims: 7-9, 12 and 1-3, 10, 13-22 (partially)

UDP-D-xylose proteoglycan core protein
beta-D-xylosyltransferase II (XT-II) and its use

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